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Characterization of Biofilms Formed by Mycobacterium aviumn subspecies paratuberculosis in Psychologically Relevant Conditions

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Characterization of Biofilms
Formed by
Mycobacterium avium
subspecies *paratuberculosis*
in Physiologically Relevant Conditions

By
Richard John Brunner

A Thesis Submitted in Partial fulfillment of the Requirements for
Master of Science
In
Biology

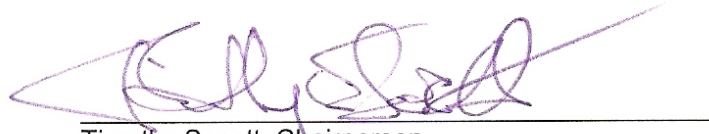
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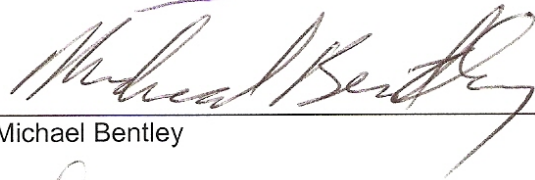
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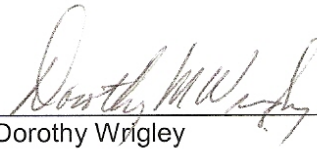
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Table of Contents

Chapter One: Introduction.....	1
Chapter Two: Literature Review	4
History of Johne's disease	4
Related species	5
Characteristics of <i>Mycobacterium</i>	5
Economic impact of Johne's on dairy herds	6
Diagnosis	7
Transmission of <i>M. paratuberculosis</i>	8
Pathology	9
Survival of Mpt in the Environment	10
Biofilms	10
Biofilm development.....	11
Benefits of Biofilms	12
Mycobacterial Biofilms	13
Summary.....	14
Chapter 3: Materials and Methods	15
Bacteria	15
Media	15
M7H9C.....	15
M7H9C ΔpH.....	15
M7H9C Oxyrase™.....	15
MSUM1	16
MSUM1 with Hydrogen Peroxide	16
Culture containers and conditions	16
24-well plates	16
Thermanox Coverslips.....	16
24-well Plate-Thermanox Discs Assembly	16
Incubation chamber	17
Mpt in M7H9C ΔpH.....	17
Mpt in M7H9C with Oxyrase™	18
Mpt in MSUM1 with Hydrogen Peroxide.....	19
Assays	19
Viability Assay.....	19
ATP Assay	20
Biomass Assay	21
Analysis.....	22
Viability Assay.....	23
ATP Assay	23
Biomass Assay	23
Biofilm Index	23
Two-way Analysis of Variance.....	24

One-way Analysis of Variance.....	24
Chapter 4 Results	30
Section 1: Characterization of the effects of pH level on Mpt biofilms grown in M7H9C	30
Viability Ratio	30
SYTO 9 Staining	30
Propidium Iodide Staining.....	31
Cellularity	31
ATP Assay	32
Biomass Assay	33
Biofilm index	33
Section 2: Characterization of the effects of oxygen deprivation on Mpt biofilms	34
The effects of Oxyrase™ on oxygen levels over time in an open system.....	34
Viability ratio	34
SYTO 9 staining.....	35
Propidium Iodide staining	35
Cellularity	36
ATP Assay	36
Biomass Assay	37
Biofilm Index	37
Section 3: Characterization of the effects of oxidative stress on biofilms of Mpt	38
Viability Ratio	38
SYTO 9 Staining	39
Propidium Iodide Staining.....	39
Cellularity	40
ATP Assay	41
Biomass Assay	42
Biofilm Index	42
Chapter 5: Discussion	65
The Effects of pH on Biofilm Development.....	66
Iron and pH Level	68
Environmental pH levels	68
The Effects of Oxidative Stress on Biofilm Development	70
Biomass Assay	72
Viability Assay.....	73
ATP Assay	74
Biofilm Index	76
Closing Comments.....	76
List of Figures.....	78
References.....	79

Abstract

Mycobacterium avium subspecies *paratuberculosis* (Mpt) is the cause of Johne's disease, a gastrointestinal disease that mainly affects ruminants. Despite being an obligate intracellular pathogen, Mpt can survive in the environment for months. How Mpt survives in the environment has yet to be determined, but one tactic Mpt may use is the formation of biofilms. Biofilms may provide sufficient protection and allow Mpt endure in the environment until a new host is encountered. The conditions affecting Mpt biofilm formation have not been investigated. Both in the host and in the environment, Mpt experiences physiological stresses that may induce biofilm formation including suboptimal pH, oxidative stress and hypoxic stress. Mpt was subjected to various levels of pH, oxidative stress or hypoxia and cultured for 20 or 30 days. Adherent biofilms were characterized for biomass, viability, cellularity and metabolic activity. Biofilm biomass was measured by staining biofilms with crystal violet, destaining with ethanol and measuring the crystal violet-ethanol solution for optical density. Viability and cellularity were measured using the fluorescent DNA dyes SYTO 9 and propidium iodide to differentially stain cells based on membrane integrity. Metabolic activity was assessed using a bioluminescence reaction to measure ATP. Under standard conditions for culturing Mpt, biofilms were the most robust. Alkaline conditions inhibited biofilm formation. Oxidative stress limited biofilm formation. Hypoxic shock may have forced Mpt into early stationary phase or dormancy. In conclusion, Mpt will form single species biofilms and do so under conditions optimal for planktonic growth in vitro.

Chapter One: Introduction

Mycobacterium avium subspecies *paratuberculosis* (Mpt) causes an intestinal disease in ruminants known as Johne's disease (9). In a 1996 study by the United States Department of Agriculture, Johne's disease was estimated to cost the United States dairy industry \$200 to \$250 million per year due to loss of production and early culling of infected animals (36).

Johne's disease is typically transmitted through a fecal-oral route (22). Animals are typically infected in the first six months of life, but generally do not display clinical signs of Johne's disease until two to five years after initial infection (7). After ingestion, Mpt cells are taken up through the M-cells in the ileum. Macrophages engulf Mpt cells and sequester the cells in granuloma. Mpt is capable of surviving and thriving within ruminant macrophages. As Johne's disease progresses, the microvilli of the intestines become flattened. This limits nutrient absorption, leading to malnutrition, emaciation, and death. While the animal is not displaying clinical signs of the disease, Mpt cells are shed into the environment. As Johne's disease becomes more severe, the quantity of cells shed increases.

Although considered to be an obligate intracellular pathogen, Mpt can survive in the environment for up to a year (54). Whittington et al studied the survival of Mpt in the environment and found that under the best conditions, Mpt could survive for 55 weeks outside of a host in a farm field.

There are several hypotheses regarding the means of survival of Mpt in the environment, including intermediate hosts, dormancy and biofilms (39). Biofilms are communities of microorganisms surrounded by an extracellular matrix and often adhere to surfaces (14). The extracellular matrix provides protection to resident bacteria. Cells encased in an extracellular matrix are more resistant to antibiotics and disinfectants than their planktonic counterparts. Therefore biofilms can allow pathogens to survive in hostile environments longer than planktonic cells (47).

Richards collected anecdotal data suggesting that the pH of the soil may affect survival of Mpt in the environment (38). He observed that Johne's disease was prevalent on farms with

acidic soil, but not on farms with alkaline soil. He noted further that a Missouri farmer was able to eradicate Johne's disease from his farm through liming, which raised the pH of the soil. Richards speculated that the pH of the soil may influence iron acquisition by Mpt. It may be that soil acidity induced biofilm formation, allowing Mpt to survive longer and infect more animals than it would in alkaline soils.

Secott et al observed that Mpt formed biofilms in response to hypoxic conditions while investigating the effects of hypoxia on dormant Mpt (40). Secott used Oxyrase™, a commercially available product that removes oxygen from media, to slowly return the dormant cells to normal oxygen levels. While cultures were being put into a dormant phenotype, by incubating Mpt for extended periods in increasingly hypoxic conditions, Secott et al. observed that Mpt formed biofilm-like structures..

While investigating *Mycobacterium avium* subspecies *avium* (Maa) biofilms, Geier et al demonstrated that Maa forms biofilms in response to oxidative stress (19). When treated with 50 mM hydrogen peroxide, Maa formed biofilms with a greater biomass than when not treated with hydrogen peroxide. Since Maa and Mpt are nearly genetically identical, it is reasonable to expect that Mpt would respond like Maa to oxidative stress.

Hypotheses

Given these observations, we hypothesized that stress may induce biofilm formation by Mpt.

- Mpt forms biofilms poorly in alkaline conditions
- Mpt forms biofilms best with an hydrogen peroxide concentration of 0.01% H₂O₂
- Mpt forms biofilms best in the presence of sub-ambient oxygen concentrations

To test these hypotheses, Mpt was cultured in several different media in 24 well microtiter plates. Each well of the microtiter plates had a Thermanox coverslip to standardize the surface area that biofilms would adhere to. To test if Mpt forms biofilms poorly in alkaline conditions, media were adjusted with HCl and NaOH to pH of 6 to 8. To test if Mpt forms biofilms best with a hydrogen peroxide concentration of 0.01% H₂O₂, media with hydrogen peroxide concentrations of 0.1% H₂O₂, 0.01% H₂O₂ and 0.001% H₂O₂ were prepared. To test if Mpt forms biofilms best in

the presence of sub-ambient oxygen concentrations, media was prepared with Oxyrase™ to remove the oxygen from the media. .

To characterize biofilms, three assays were selected. The biomass assay described the amount of cells and extracellular matrix. The viability assay used nucleic acid dyes to differentially stain cells based on cell viability. The metabolic activity assay used a bioluminescence reaction to determine the ATP content in biofilms.

The typical biofilm assay uses crystal violet to measure the biomass of biofilms. In a biomass assay developed by O'Toole et al, bacteria were cultured in a 96 well microtiter plate, and then the wells were stained with crystal violet and washed with water (33). Crystal violet binds to many macromolecules including cells and the extracellular matrix. Crystal violet is solubilized with ethanol and the solution is measured for optical density. The darker the solution, the more biomass was present from adherent biofilms.

The viability assay used Live/Dead™ stain, a set of fluorescent nucleic acid dyes that differentially stain cells based on cell membrane integrity. Cells with intact cell membranes fluoresce green. Cells without intact cell membranes fluoresce red. The red and green fluorescence signals represent the relative amount of live and dead cells in a biofilm.

Metabolic activity was measured using BacTiter Glo™, an ATP assay based on the bioluminescence of luciferin-luciferase reactions. The level of ATP can be used as a measure of metabolic activity. Cells that are metabolically active have high ATP concentrations. Cells that are in stationary phase or dormancy have lower ATP concentrations.

A biofilm index was developed to incorporate these assays into one description of biofilms. Data were normalized to give equal weight to each assay. The biofilm index describes which conditions were best for Mpt biofilm development in terms of cellularity, viability, biomass and metabolic activity.

Chapter Two: Literature Review

Paratuberculosis, commonly called Johne's disease, is a gastrointestinal disease that mainly affects ruminants such as cows, sheep, camels, llamas and wildebeest (22). *Mycobacterium avium* subspecies *paratuberculosis* (Mpt) the organism that causes Johne's disease has also been linked to Crohn's disease, a human intestinal disease (6). According to a 1996 USDA study, Johne's disease costs the US dairy industry \$200 million to \$250 million annually (36). Despite being an obligate intracellular pathogen, Mpt can survive in the environment for months (54). How Mpt survives in the environment has yet to be determined, but one tactic Mpt may involve the formation of biofilms (39).

Since the initial descriptions of Johne disease were published, some progress in understanding Mpt and Johne's disease has been made. Governments, scientists and economists continue to study the economic impact of Johne's disease on the agriculture industry (36). Genetic studies of the *Mycobacterium* genus have determined the genetic differences between *M. avium* subspecies *avium* and *Mycobacterium avium* subspecies *paratuberculosis* (29). The structure and function of mycobactin, a siderophore essential for iron acquisition in Mpt metabolism has been determined (42). In general terms, we understand the transmission and pathology of Johne's disease but the specifics aspects remain to be elucidated (22). Since transmission of Mpt is mainly through a fecal oral route, determining how Mpt survives in the environment would be beneficial to preventing the spread of Johne's disease.

History of Johne's disease

In 1895 Heinrich Johne and Langdon Frothingham described a gastrointestinal disease in cattle later called Johne's disease. During postmortem inspection of a cachectic cow that had had persistent diarrhea, Johne and Frothingham discovered acid-fast bacilli in the ileal tissues. However, they were unable to satisfy Koch's postulates in the late nineteenth century to prove that Mpt was the causative agent of Johne's disease (27).

In 1912, F. W. Twort managed to culture Mpt from the intestinal tissues of cattle with Johne's disease and thereby meet the second of Koch's postulates, leading to proof that Mpt was the cause of Johne's disease (9, 22, 52). Twort decided that the media used for *Mycobacterium*

tuberculosis and *Mycobacterium phlei* culture lacked some ingredient that was needed for growth of Mpt. Twort was able to culture Mpt by culturing Mpt with heat-killed *M. phlei* (52). The missing ingredient provided by killed *M. phlei* was later identified as mycobactin. Mycobactin is a complex organic molecule that binds ferric iron and plays a role in transporting iron into the cell (43). Unlike *M. tuberculosis* and *M. phlei*, Mpt is unable to produce mycobactin.

Related species

Some notorious pathogens are closely related to Mpt. The causative agent of tuberculosis, *M. tuberculosis* (Mtb), is an intracellular pathogen typically found in macrophages in the lungs. The causative agent of leprosy, *M. leprae*, causes nerve damage. An opportunistic pathogen, *M. avium* subspecies *avium* (Maa) often infects immunocompromised patients and is very closely related to Mpt. Like *M. tuberculosis* and *M. leprae*, Mpt is an intracellular pathogen (1).

As part of the *Mycobacterium avium* complex, *M. avium* subspecies *paratuberculosis* and *M. avium* subspecies *avium* are nearly genetically identical. Phenotypically, Mpt differs from the other subspecies of *M. avium* in that Mpt requires the addition of mycobactin to grow *in vitro*. The other subspecies of *M. avium* are capable of synthesizing mycobactin. Mpt lacks functional genes to produce mycobactin. Genotypically, Mpt also differs from Maa in that the genome of Mpt contains IS900, an insertion sequence unique to Mpt (22).

Characteristics of *Mycobacterium*

A defining characteristic of the genus *Mycobacterium* is the waxy cell wall made of mycolic acid, a very hydrophobic fatty acid, which helps them survive in environments that would kill other species of bacteria. The low permeability of the cell wall limits desiccation and limits the penetration of antibiotics and disinfectants. Mycolic acid in the cell confers acid-fastness to *Mycobacterium* species. Acid-fast bacteria resist decolorization by acid alcohol of the Ziehl-Neelsen acid-fast staining process and retain the bright pink of carbol-fuchsin, the primary stain. Non-acid-fast bacteria are unable to resist the acid alcohol decolorization step and are counter-stained with methylene blue (25).

The thick waxy mycolic acid cell wall provides great protection to mycobacteria, but the low permeability of the cell wall also slows growth. Bacteria like *Escherichia coli* grow quickly, having generation times that can be measured in minutes. *Mycobacterium* species have generation times that are measured in hours or days. Part of the reason they are slow growers is that the cell wall limits the amount of nutrients that can be brought into the cell (25).

Economic impact of Johne's on dairy herds

A 1996 USDA study estimated that the cost of Johne's disease to the US dairy industry was \$200 million to \$250 million annually (36). Although the economic impact of paratuberculosis on the United States cattle industry has not been definitively determined, the cost of Johne's disease has been estimated to exceed \$1.5 billion per year (45). Much of the economic damage to the dairy industry comes from the loss of milk production and early culling of infected animals.

There are several economic models to derive the cost of Johne's disease. Some of the more complicated models include international trade into the equation. A simple model proposed by L. Hasonova and I. Pavlik looked at economic losses from the farmer's viewpoint (23). In the Hasonova-Pavlik model, economic cost is defined by loss of production, treatment costs and prevention costs.

Cows afflicted with Johne's disease produce less milk, produce fewer offspring and have a shorter life expectancy than healthy cows. The amount of milk produced by a healthy cow is greater than a cow infected with Johne's disease (2). Sick cows are culled from the herd earlier than healthy cows. Calves are less likely to be carried to term by sick cows than healthy cows, decreasing the number of replacement animals over the lifetime of a cow. At the end of their productive lives, healthy cows are worth more than unhealthy cows when sold to a slaughterhouse. Some cattle in the clinical phase of Johne's disease may have no slaughter value.

Treatment of Johne's disease is difficult, cost prohibitive and largely ineffective. Clinically diseased animals would require daily medication to halt the progress of Johne's disease. On a farm with hundreds of animals, treating animals with Johne's disease is not feasible, as the cost

to treat sick animals would exceed the value of the animal. Culling sick animals from the herd is a far less expensive alternative to treatment of Johne's disease.

Preventative costs include separating sick animals from healthy animals and keeping healthy animals healthy. Some of prevention costs relate to testing to determine which animals have Johne's disease. Since the lag from infection to clinical signs of disease is 2 to 5 years, infected animals without clinical signs of Johne's disease can only be identified through laboratory tests. Subclinical infection with Mpt can be diagnosed through serological tests or through culturing Mpt from feces, but these tests are expensive to perform on every animal on the farm. In addition, animals may spread Mpt in their feces before clinical signs of Johne's disease are present.

Diagnosis

Diagnosis of Mpt infection is accomplished mainly through three methods: fecal culture for Mpt, detection of anti-Mpt antibodies in the serum, and detection of DNA sequences unique to Mpt. Fecal culture is the "gold standard" for diagnosing Johne's disease. However, fecal culture is laborious and takes several weeks to complete. Serological tests are quick but may not detect Johne's disease in the early stages of disease. DNA probes also have a quick turnaround but require technical expertise.

Culturing Mpt from feces can take months and isolating any single species from feces is difficult at best due to the large and diverse population of bacteria in feces (44). Mpt is a very slow growing organism and contamination during the culturing on agar often occurs before colonies appear (41). Mycobactin dependence is a simple method to confirm the isolate is Mpt. If the bacteria grow only in the presence of mycobactin, the bacteria are identified as Mpt. Other more technical tests can be used to prove the presence of Mpt, such as DNA probes and polymerase chain reaction (PCR) to show that genetic material from Mpt is present in the isolated organism (41).

Serologic tests are less sensitive, but are much faster than fecal culture (8). Enzyme-linked immunosorbent assay (ELISA) testing for the presence of antibodies for Mpt in the serum

is currently the most sensitive serological test for Mpt (22). About 30–40% of cattle identified as positive by fecal culture are also identified as positive with ELISA (10). The main problem with using ELISA to detect Mpt is that antibodies are often not produced until later in the pathology of Johne's disease (7).

DNA probes for Mpt can be performed with three different sources: fecal matter, tissue and cultured bacteria. DNA assays on fecal matter are difficult to perform due to the small quantity of Mpt compared to the large quantity of fecal matter and non-Mpt bacteria. Using tissue from infected animals is difficult to acquire while the animals are still alive, but is effective in postmortem testing. Using a blend of culturing Mpt and DNA probes has been shown to be effective. Suspected Mpt cultured from feces can be tested for Mpt specific DNA using DNA probes or PCR. This takes longer than testing the feces but is more specific (41).

Transmission of *M. paratuberculosis*

Studies have shown that Mpt can be transmitted through milk (49), semen (24), and from mother to calf *in utero* (55). Mpt is known to be present in milk and has even been detected in pasteurized milk (21). However, the most studied and likely the most prevalent form of transmission in cattle is fecal-oral transmission (45).

Animals with subclinical infections shed fewer Mpt cells in feces than animals with clinical Johne's disease (22). As Johne's disease progresses, the quantity of bacteria shed increases. Expecting farms to be as clean as an operating room is unreasonable; however, some common farm practices lead to cross-contamination. When animals infected with Mpt share the same space at different times with uninfected animals, Mpt could be transmitted to uninfected animals. Using the same bucket loader in Mpt contaminated areas and areas for uninfected animals may be a means of cross contamination (53).

One of the major problems with defeating Johne's is the long delay between infection and diagnosis. Animals typically do not develop clinical signs of the disease until 2 to 5 years post infection (9). Meanwhile as subclinically infected animals carry Mpt, they may shed Mpt in feces posing the risk of infecting other animals in the herd. Often animals are not identified as carriers

of Mpt until long after the animal was initially infected. Since subclinically infected animals shed Mpt, these seemingly healthy animals may be spreading Mpt to the uninfected animals.

A secondary route of transmission is from the milk of infected cows. Cows without clinical signs of Johne's disease can shed 1 to 16 Mpt cells per 100 ml of milk (21). While 16 Mpt cells per 100 ml of milk is a relatively low concentration of bacteria, this may be sufficient to infect nursing offspring. Several studies have shown that Mpt can survive pasteurization of milk (49). Mpt has been cultured from milk taken off the shelf of grocery stores. Some studies have implicated Mpt as a cause of Crohn's disease, this point is very controversial (3, 4, 6). If Mpt is a cause of Crohn's disease, milk could serve as a vehicle for transmission to humans.

Pathology

Clinical signs of Johne's disease begin with intermittent diarrhea. As the disease progresses, diarrhea becomes progressively more severe and more consistently present. Along with diarrhea, milk production decreases and infected animals begin to lose weight. Intestines of infected animals are damaged as Johne's disease progresses. Inflammation causes flattening of the microvilli, which restricts the absorption of nutrients from the intestines. In the late stages of Johne's disease, animals are cachectic, appearing thin and unhealthy (22).

Once in the digestive tract, Mpt is taken up by the microfold cells (M cells) in the intestines. The Mpt is then transferred to macrophages. While macrophages are able to kill most bacteria, Mpt can survive the oxidative burst and other microbial digestion efforts of the macrophage. Within the macrophages Mpt not only survives but grows (7).

Necropsy of affected animals most often reveals primary lesions in the ileum. Secondary infection sites include other portions of the intestines and lymph tissue near the ileum. Tertiary infection sites include systemic immune tissues such as the spleen, lymph nodes and the liver (7).

Before clinical signs of Johne's disease appear, Mpt is mainly sequestered in the phagosomes of macrophages near the ileum. In the subclinical phase of Johne's disease the immune response is largely cell mediated. Subclinically infected animals often do not have

antibodies for Mpt in sufficient quantities to be detectable with serologic tests. Since most if not all of the Mpt are sequestered in granulomas, most of the subclinically infected animals are also fecal culture negative (7).

Clinical signs usually appear two to five years after the animal is first infected with Mpt. As Johne's disease progresses, granulomas enlarge and macrophages become packed with Mpt cells. For an unknown reason, the balance of the immune response transitions from cell mediated to humoral. The humoral response exacerbates intestinal inflammation and intestines become flattened which decrease the surface area of the intestines (7).

Survival of Mpt in the Environment

Whittington et al have looked into the survival of Mpt in the environment and has been shown to survive for up to a year outside of a host (54). Subclinically and clinically infected animals are known to shed Mpt in the environment, which serves as a reservoir for infecting animals (22). How Mpt survives in the environment outside of a host is unknown. Since Mpt is considered an obligate intracellular pathogen and requires mycobactin to grow *in vitro*, replication in the environment would be expected to be very limited.

There are several hypotheses regarding Mpt can survival in the environment between hosts (39). Mpt may go into a dormant phase, where most metabolic activates cease. Mpt may infect an intermediate host such as protozoa or insects. It is also possible that Mpt may form a biofilm to survive in the environment until a new host is encountered.

Biofilms

Biofilms are a community of single-celled organisms surrounded by an extracellular matrix and attached to a surface. "Surface" could mean anything from an abiotic surface like a rock to the biotic surface of a tooth to the liquid-air interface of a pond. Macroscopically, biofilms form as a sheet on a surface. Microscopically, biofilms can have a complex architecture analogous to multi-cell organisms. Biofilms can protect resident bacteria from physical or

chemical harm, such as desiccation and antimicrobial agents. Thus the microenvironment provided by biofilms allows bacteria survive in less hospitable niches.

Biofilms are many individual single-celled organisms surrounded by an extracellular matrix, which is the glue that holds the community together and holds the community to the surface. Composition of the matrix is dependent on the species in the biofilm and could be composed of polysaccharides, proteins, lipids or combinations of these (57). The extracellular matrix not only holds the biofilm together but protects the cells living within the biofilm from physical damage and from antimicrobial agents and disinfectants (20).

In a natural setting, biofilms are usually composed of many species and sometimes different kingdoms. Most of the bacteria in the world live in biofilms. Free-living, planktonic bacteria make up only a small fraction of all the bacteria on earth (14). The best known bacterial biofilm is dental plaque, which can contain hundreds of bacterial species (37). Occasionally biofilms composed of a single species can be seen in a natural setting such as those formed by *Pseudomonas aeruginosa* in people with cystic fibrosis (15).

Biofilm development

Biofilms develop in three main phases, beginning with planktonic bacteria and ending with a mature biofilm encased in an extracellular matrix (12). Biofilms begin with planktonic bacteria that adhere to a surface. The first cells divide to form a microcolony of a few cells. As the microcolony matures into a biofilm, an extracellular matrix of lipids, proteins and/or polysaccharides surrounds the cells.

The first phase of biofilm development begins with a surface and planktonic bacteria. Planktonic bacteria transiently adhere to surfaces. Bacteria use several mechanisms to attach to surfaces. Some organisms use pili, fimbriae or flagella to hold onto a surface. Other bacteria use a glycocalyx or capsule to stick to a surface. If conditions are favorable, the bacteria will grow and proceed to form a biofilm (12).

After the first bacteria attach to a surface the process of forming a biofilm begins. The number of cells increases through cell division and in some species through chemical signaling to

recruit more planktonic bacteria to join the forming biofilm. As the biofilm begins to mature, the pioneer cells transform from a monolayer into a microcolony that has a vertical element as cells begin to pile up on the surface (12).

The microcolony is a transition state, as planktonic bacteria become biofilm residents. A main difference between a biofilm and an aggregate of cells is the extracellular matrix. As the aggregate of cells in the microcolony transition into a biofilm, an extracellular matrix is exuded to surround the cells. The extracellular matrix helps the cells adhere more firmly to the surface. Mature biofilms often have a more complex architecture than just a pile of cells. In some biofilms, channels are formed to allow for the flow of fluids, which allow for the ingress of nutrients and the egress of waste (16).

Benefits of Biofilms

Most bacteria live in biofilms rather than as planktonic bacteria due to the benefits that biofilms provide (14). Living in a biofilm allows bacteria to stay in one place without using energy to stay there. In multi-species biofilms, the waste of one species can be the nutrients of another (12). The close-quartered, sessile environment of a biofilm is beneficial for sharing DNA (28). Biofilms provide protection from chemical and physical damage (16).

There are two general hypotheses regarding how the extracellular matrix protects bacteria from chemical threats: lack of penetration and phenotypic resistance. Some chemicals do not penetrate to the basement of the biofilm. In certain species, the cells within the biofilm are phenotypically different than planktonic cells and are more resistant to certain chemicals. Each of these hypotheses has merit and the species involved influence which hypothesis is correct (47).

The extracellular matrix may limit the penetration of chemicals into the microenvironment at the bottom of the biofilm. Some antimicrobial molecules are just too large to fit through the net formed by the polymer of the extracellular matrix. Some antimicrobial molecules may be bound to the extracellular matrix through ionic attraction or hydrophobic interaction. Some antimicrobial agents may react with the extracellular matrix, rendering the antimicrobial agent ineffective (46).

Some bacteria change phenotypically when living within a biofilm. The biofilm slows growth of cells within the biofilm by limiting nutrients and oxygen. Cells within the biofilm may transition from an exponential growth phase phenotype to a stationary phase phenotype. Slow growth can limit the effectiveness of some antibiotics (30).

Mycobacterial Biofilms

Like many other genera of bacteria, *Mycobacterium* species bacteria are capable of forming biofilms. Under certain conditions *M. tuberculosis* forms a biofilm *in vitro* and possibly *in vivo* leading to antibiotic resistance (35). *M. avium*, a common opportunistic pathogen in AIDS patients, is often found in biofilms in water pipes (51). Mpt is capable of forming biofilms in cattle water troughs (11).

Ojha et al proposed that *M. tuberculosis* may form biofilms *in vivo* (35). Biofilms may allow *M. tuberculosis* to persist through the first few weeks of treatment, necessitating treatment for months in order to clear *M. tuberculosis* from the body. Biofilms increase the resistance to antibiotics either through limiting the concentration of antibiotics reaching cells or by reducing the effectiveness of the drugs due to phenotypic changes in cell physiology. *M. smegmatis*, a saprophytic organism and a work-horse of *Mycobacterium* research is less resistant to Isoniazid, a common anti-tuberculosis drug, as planktonic bacteria than when part of a biofilm community (50).

Often found in biofilms in water and sewage pipes, *M. avium* (Maa) is an opportunistic pathogen that can infect people with compromised immune systems. Maa is nearly identical genetically to Mpt. Since Maa can form biofilms, Mpt likely uses similar mechanisms to form biofilms (5).

Cook et al studied Mpt in mixed species biofilms in cattle watering troughs. Using trough water from active farms as a source of normal flora bacteria, Cook examined several trough materials for biofilms. Trough water was spiked with Mpt in some replicates to determine if Mpt could survive within a mixed community biofilm. In a mixed community biofilm, Mpt was able to survive for weeks (11).

Summary

Since the time of Twort, some progress has been made on understanding Mpt and Johne's disease (9). Some of the pathology and physiology of Mpt is understood (7). Detection techniques are not effective enough detect Mpt early enough to cull animals before they can pass Mpt to other animals (34). Current vaccines are ineffective. Current antibiotic treatments are too costly to be practical (9). The understanding of how Mpt survives in the environment between hosts is incomplete, making prevention ineffective (39). Transmission of Mpt from animal to animal is partially understood (9). A solution to eradicate Mpt from cattle, goat and sheep herds remains elusive. Understanding how Mpt survives in the environment may improve efforts to prevent Johne's disease or to eradicate Johne's disease.

Chapter 3: Materials and Methods

Bacteria

Mycobacterium avium subspecies *paratuberculosis* PAMSUM 8 is a strain of bacteria recovered from a cow in Pennsylvania.

Media

M7H9C

M7H9C is a derivative of Middlebrook 7H9, which is used to culture many species of *Mycobacterium*. M7H9C was composed of Middlebrook 7H9 supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC), 0.005% Tween 80 and 2 µg/ ml mycobactin J. The bovine serum albumin (BSA) in OADC makes the solution heat labile and must be added after autoclaving.

M7H9C ΔpH

Normal pH for M7H9C is pH 6.8. The pH was adjusted after OADC was added to ensure that adding OADC after pH adjustment did not alter the pH. The pH was measured with a pH probe calibrated to pH 7.00. M7H9C was poured into an Erlenmeyer flask containing a magnetic stir rod. The flask was placed on a magnetic stirrer and the pH probe was placed so that the probe was about 1 cm below the surface of the medium. Either HCl or NaOH was added while the medium was stirred slowly until the desired pH reached. The M7H9C was filter sterilized using a 0.22 µm pore filter. Filtered M7H9C was stored in a 37°C incubator overnight to check for contamination.

M7H9C Oxyrase™

Recommended strength for Oxyrase™ is 20 µl Oxyrase™ per 1 ml broth to achieve anaerobiasis according to the manufacturer. M7H9C was used as the base medium for this experiment. Moments before inoculation with Mpt, Oxyrase™ was diluted with M7H9C in two-fold serial dilutions to concentrations of 1/4, 1/8, 1/16 or 1/32 of full strength, e.g. 25 µl Oxyrase™ per 5 ml medium for 1/4 strength.

MSUM1

M7H9C is not compatible with hydrogen peroxide experiments because the OADC in M7H9C contains catalase. Rather than removing catalase from OADC, a similar supplement, ADS containing BSA, dextrose and NaCl in the same ratios as in OADC was used to enrich Middlebrook 7H9 broth base to make MSUM1. MSUM1 was supplemented with Tween 80 and mycobactin J in the same proportions as M7H9C.

MSUM1 with Hydrogen Peroxide

A stock solution of 30% hydrogen peroxide was diluted to a 1% working dilution using MSUM1. Immediately before inoculation, 1% hydrogen peroxide in MSUM1 was serially diluted into the medium to a final concentration of 0.1%, 0.01% or 0.001% hydrogen peroxide. Hydrogen peroxide was diluted into MSUM1 moments before the test plates were inoculated with Mpt to limit any effect time and temperature might have on hydrogen peroxide. A 0.1% hydrogen peroxide solution equates to 32.6 mM.

Culture containers and conditions

24-well plates

The base growth platform for these experiments was polystyrene, gamma irradiated 24-well microtiter plate that was not coated with any cell adhesion chemicals (Corning, Corning NY). The lid for the 24-well plate was designed to allow for airflow, and did not seal the plate.

Thermanox Coverslips

Thermanox coverslips (Thermo Fisher Scientific Inc, Waltham, MA) are coated on one side to promote cell adhesion and are used for eukaryotic cell culture. Because the surface area of a well may change for biofilm growth over time as the medium evaporates and condenses. Discs were placed in the bottom of each well of a 24-well plate to standardize the growth surface area.

24-well Plate-Thermanox Discs Assembly

Thermanox coverslips were and 24-well plates were received sterile. Autoclaved forceps were used to transfer discs from the packaging to the wells of the 24-well plate. Hands and gloves were sanitized with 70% ethanol. All culture assembly and manipulation was conducted in

a laminar flow hood. Coverslips were transferred from their packaging to the wells one by one touching them only with the sterile forceps.

After all of the discs were placed in the 24-well plate, the wells were flooded with 3% hydrogen peroxide and allowed to incubate for 30 minutes. The wells were then emptied with a sterile Pasteur pipette connected to a vacuum pump. Because hydrogen peroxide tended to become trapped under the disc during the emptying process, the discs were thoroughly dried in a 37°C incubator for a minimum of 48 hours to allow the hydrogen peroxide to evaporate. Complete removal of detectable hydrogen peroxide was verified by potassium permanganate colorimetric dye titration (26).

Incubation chamber

In these experiments, Mpt grew over several weeks. The 24-well plates used to culture Mpt allowed for air to circulate, which could have led to evaporation if the system was not sealed. (Figure 3-2) Cheesecloth soaked in Roccal increased the humidity within the container, limiting the amount of evaporation of the media and limiting the growth of contaminants.

Containers were cleaned with Alconox and allowed to air dry. Cheesecloth was cut to fit in the bottom of the container. Roccal was poured into the container to dampen the cheesecloth. The container lid was placed loosely over the container bottom. The container was then autoclaved for 15 min at 121°C and 15 pounds per square inch. The autoclave was cooled to room temperature to prevent the container from collapsing. The containers were quickly sealed and moved to the laminar flow hood. After the microtiter plates were filled with the appropriate medium and inoculated with Mpt, the plate was placed in the airtight container. The sealed airtight container was then placed in an incubator at 37°C.

Mpt in M7H9C ΔpH

The base medium for this experiment was M7H9C. The pH was adjusted with HCl and NaOH to pH 6.0, 6.5, 6.8, 7.0, 7.5 or 8.0. After pH adjustment media were filter sterilized through a 0.22 μm pore filter. The unadjusted pH level for M7H9C is pH 6.8 (Figure 3-2).

Mpt was grown to mid-logarithmic phase in M7H9C pH 6.8. The cells were aseptically poured into a 50 ml centrifuge tube and centrifuged at 2000 RCF for 30 minutes. The pellet was resuspended in 10 ml sterile phosphate buffered saline with Tween 80 (PBST). The optical density of Mpt was adjusted to 30% transmittance at 590 nm, which is equivalent to 2×10^8 CFU/ml (40). The suspended cells were then centrifuged again and resuspended in M7H9C.

Each well of 24-well microtiter plates containing coverslips was filled with 1 ml of pH adjusted M7H9C (Figure 3-2). Each well was inoculated with 10 μ l of Mpt, making the final concentration of Mpt approximately 10^5 CFU/ml. After inoculation plates were sealed in plastic containers and incubated for 20 or 30 days. After 20 or 30 days biofilms adhering to coverslips were removed from the plate using forceps and placed into fresh microtiter test plates. Each sample was subjected to one of three assays: biomass, viability or ATP.

Mpt in M7H9C with Oxyrase™

The base medium for this experiment was M7H9C. Oxyrase™ was serially two-fold diluted in M7H9C to concentrations of 1/ 2, 1/ 4, 1/8 and 1/16. Dilutions of M7H9C with Oxyrase™ were prepared so that when combined with an equal volume of 2×10^5 CFU Mpt/ ml suspension, the Oxyrase™ content would be 1/4 Oxyrase™, 1/8 Oxyrase™, 1/16 Oxyrase™, or 1/32 Oxyrase™ and the bacterial concentration would be 1×10^5 CFU Mpt /ml (Figure 3-3)

Mpt was grown to mid-logarithmic phase in M7H9C and cells were prepared as above for the pH experiment except that the culture was then diluted to 2×10^5 CFU/ml in M7H9C. Oxyrase™ in M7H9C at half strength was produced by diluting 250 μ l Oxyrase™ into 25 ml M7H9C. Oxyrase™ at half strength was then serially diluted by half dilutions to 1/16th strength Oxyrase™. To each 50 ml centrifuge tube, 12.5 ml of M7H9C with Mpt was added. These four treatments were then dispensed into 24-well plates. (Figure 3-3) After inoculation growth plates were sealed in plastic containers and incubated for 20 or 30 days. After 20 or 30 days biofilms adhering to Thermanox coverslips were extracted from the growth plate using forceps and placed into fresh microtiter test plates. Each replicate was measured with one of three assays: biomass, ATP or viability.

Mpt in MSUM1 with Hydrogen Peroxide

The base medium for this experiment was MSUM1. A stock solution of 30% hydrogen peroxide was diluted to 1% hydrogen peroxide with MSUM1. The media was prepared by serially 10-fold diluting 1% hydrogen peroxide to 0.1% H₂O₂, 0.01% H₂O₂ and 0.001% H₂O₂ in MSUM1. (Figure 3-4)

Mpt was grown to mid-logarithmic phase in MSUM1 and cells were prepared as above for the pH experiment except that the culture was resuspended in MSUM1. Each well of 24-well microtiter plates with Thermanox discs aseptically placed in the bottoms of the wells were filled with 1 ml of MSUM1 with or without hydrogen peroxide. Each well was inoculated with 10 µl of Mpt in MSUM1 at 2×10^8 CFU/ml, making the final concentration of Mpt approximately 10^5 CFU/ml. After inoculation growth plates were sealed in plastic containers and incubated for 20 or 30 days. After 20 or 30 days biofilms adhering to Thermanox coverslips were extracted from the growth plate using forceps and placed into 24-well microtiter plates. Each replicate was examined using biomass, ATP or viability assay.

Assays

Three different assays were chosen to characterize the biofilms produced by PAMSUM 8. One-third of the samples were examined with Live/Dead stain to describe the ratio of live cells to dead cells. One-third of samples were examined with BacTiter Glo to describe the amount of metabolic activity of cells in the biofilm, using a bioluminescence reaction to measure the amount of ATP present. One-third of samples were examined using a crystal violet assay to describe the amount of biomass present.

Viability Assay

LIVE/DEAD ® BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA) contains two dyes: SYTO 9 and propidium iodide. SYTO 9 penetrates all cells living and dead, and binds to nucleic acids. Propidium iodide is excluded from living cells and only penetrates cells with damaged cell membranes. SYTO 9 has moderate affinity for DNA and fluoresces green (538 nm)

when exposed to blue light (485 nm). Propidium iodide has a higher affinity for DNA than SYTO 9 (17) and fluoresces red (630 nm) when exposed to blue light (485 nm).

Biofilms of Mpt were cultured in 24-well plates for 20 or 30 days at 37°C. Discs were removed from the growth plate and placed in a black 24-well plate and stained with 200 µl 1x Live/Dead stain mix. The plates were scanned in a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific Inc, Waltham, MA). Each disc was scanned at 37 discrete locations and the sum of all these scans comprises the score for 1 sample.

ATP Assay

The ATP assay uses BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI) to measure the amount of Adenosine-5'-triphosphate (ATP) present in the biofilms (56). BacTiter Glo contains luciferin, luciferase, and a lysis agent. BacTiter Glo lyses cells, allowing the intracellular ATP to mix with luciferin and luciferase. The amount of light given off by the conversion of luciferin to oxyluciferin by luciferase is proportional to the amount of ATP present in the sample.

The lysing agent in BacTiter Glo was intended to lyse bacteria with non-acid-fast cell walls, e.g. Gram-positive *Staphylococcus aureus* or Gram-negative *Pseudomonas aeruginosa*. Early pilot studies with BacTiter Glo and PAMSUM 8 led to poor results with little luminescence. In an attempt to improve the signal, additional measures to lyse cells were studied. The best method from the pilot studies involved freezing to -80°C and thawing samples to 37°C three times in the presence of 10 mg/ ml lysozyme. At room temperature, the highest signal was within the first minute of adding BacTiter Glo to the wells. When the BacTiter Glo was added while the cells were warming up to room temp, the signal peaked several minutes after BacTiter Glo was added (Figure 3-5). Each well was read 37 times in a Fluoroskan Ascent FL (Thermo) in luminometer mode, each reading in a slightly different location within the well.

Biofilms of Mpt were cultured in 24-well plates for 20 or 30 days at 37°C. Discs were removed from the growth plates and placed in an opaque black 24-well plate. Care was taken to only transfer the disc and not biofilm that was not attached to the discs. In each well, 100 µl of 10 mg/ml lysozyme was added, which was just enough to coat the disc. The plate was then placed

in a -80°C freezer for 30 minutes. Thawing occurred in a 37°C incubator. This was repeated once, followed by a 30 minute freeze. After the third freeze cycle, 200 µl BacTiter Glo was added to each well and the plate was moved quickly to the luminometer. The plate was read twenty times consecutively in order to find the peak signal. Each sample was scanned at 37 discrete locations for bioluminescence. Each sample was scanned 20 times within an hour of the addition of BacTiter Glo. The maximum luminescence of all these scans comprises the score for one sample (Figure 3-5).

Biomass Assay

The biomass assay uses crystal violet to measure the amount of biomass in a biofilm. Crystal violet binds to many negatively charged motifs in the structure of cells and extracellular matrix. Fletcher first used this assay to measure the amount of marine bacteria adhering to polystyrene (18). The crystal violet assay was later adapted by O'Toole measure to biofilms of *Pseudomonas fluorescens* (33) and was later used for *M. avium* biofilms by Carter (5) and Geier (19).

O'Toole used the crystal violet assay in 96-well microtiter plates to measure the biofilms of *P. fluorescens* grown in various conditions. After the biofilms had developed, the media was removed from the wells, leaving only the biofilm in the wells. The wells were flooded with 2% crystal violet and allowed to incubate for 5 min at room temp. Then the wells were washed several times with tap water to remove as much of the crystal violet and loose cells as possible. Then the wells were filled with 200 µl of 95% Ethanol and allowed to sit for 5 min. Ethanol eludes the crystal violet from the cells and extracellular matrix into solution. The crystal violet-ethanol solution was transferred to a new 96-well plate and read at 570 nm in a spectrophotometer. The solution was transferred to a fresh 96-well plate to eliminate the confounding variable of biofilm cells increasing optical density (33). Fletcher tested the optical density of the biofilms rather than testing the crystal violet-ethanol solution (18).

In an attempt to enhance the contrast of the biomass assay, the O'Toole protocol was further modified. Mpt are acid-fast and crystal violet stains acid-fast bacteria poorly. *Mycobacterium* species are technically gram positive, but the mycolic acid cell wall limits the

amount of crystal violet that can penetrate the cell. Crystal violet readily flooded into the *P. fluorescens* cells and was readily removed from the cells in the presence of ethanol. In the Ziehl-Neelsen acid-fast stain, the specimen is heated up to allow carbol fuchsin to penetrate the cells. Crystal violet is molecularly similar to carbol fuchsin. By heating up the cells, crystal violet penetrated the cells better than with room temperature cells. By destaining longer and heating the cells, the optical density of the biofilms has a higher signal.

Biofilms of Mpt were cultured in 24-well plates for 20 or 30 days at 37°C. Discs were extracted from the growth plate and placed in clean 24-well plate. Each well was filled with 500 µl crystal violet. The plate was floated on a 50°C water bath for 30 minutes. After 30 minutes, the discs were washed 5 times, which is long enough to remove crystal violet not bound to the cells or extracellular matrix. About 3 ml deionized water was added to each well. Discs were lifted from the bottom with an inoculating needle to allow water to flow under the disc. The plate rested for 5 min to allow crystal violet to equilibrate within the solution. The crystal violet-water solution was removed by a Pasteur pipette attached to a vacuum pump. Care was taken to not damage the biofilm with the Pasteur pipette. Then water was added to the well and the process continued until the solution was no longer purple when the solution was removed. The wells were then filled with 500 µl Ethanol (95%) and allowed to sit for 30 min. The crystal violet-ethanol solution was transferred into a 96-well plate in four 100 µl aliquots. Occasionally the ethanol destaining step caused the biofilm to detach from the disc. By taking four aliquots the presence of cells increasing the optical density was limited. The plate was read at 570 nm in the spectrophotometer. Four wells in the 96-well plate represent one well in the 24-well plate. The four readings were averaged to give one score for that well. There were eight replicates per treatment.

Analysis

One way and two way analysis of variance was used to compare treatments. Each treatment had eight replicates. Half of the replicates were cultured at a time. Data was collected in a Fluoroskan Ascent FL (Thermo) for the viability and the ATP assays. Data was collected in a

Multiskan Spectrum (Thermo) for the biomass assays. Data was exported from the instruments in an Excel spread sheet. Data was organized in Microsoft Excel and analyzed using Graphpad Prism.

Viability Assay

The viability assay was read with a Fluoroskan Ascent FL (Thermo) fluorometer. The fluorometer scanned each well for SYTO 9 and propidium iodide fluorescence. Propidium iodide and SYTO 9 data can be analyzed several ways. The ratio of SYTO 9 fluorescence to propidium iodide fluorescence describes the relative ratio of viable cells to unviable cells. SYTO 9 data describes the relative amount of viable cells in a replicate. Propidium iodide data describes the relative amount of dead cells in a sample. A summation of SYTO 9 and propidium iodide data results in the relative amount of cells in a biofilm or cellularity.

ATP Assay

The BacTiter Glo assay measures the amount of ATP present in a sample. Each treatment had 8 replicates. Each replicate was measured twenty times in a luminometer in 37 unique locations. The 37 measurements for one reading were added together to form one score for the replicate for that reading. The maximum score out of the twenty measurements was used to compare replicates.

Biomass Assay

The biomass assay measures the amount of biomass present in the sample. Each treatment had 8 replicates. Four aliquots were taken from each replicate and were averaged to form 1 score for each replicate.

Biofilm Index

The biofilm index was devised to incorporate the biomass, viability and ATP assays into a qualitative description of biofilms. The data was normalized so that each assay has equal weight. Normalization was computed by calculating the mean for the control treatment, i.e. M7H9C pH 6.8, M7H9C without Oxyrase™ or MSUM1 without hydrogen peroxide for each assay. Each

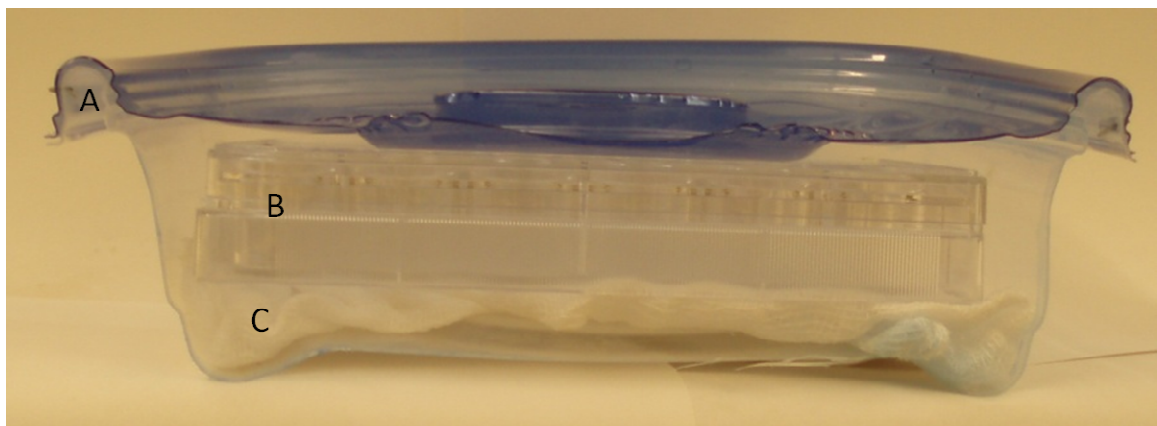
replicate in that set of treatments and assays was then divided by the mean of the control group to calculate the normalized value. For example in the pH experiment using crystal violet assay, the mean optical density for biofilms cultured in M7H9C was computed. Each of the optical densities from the other pH replicates was divided by the mean optical density for biofilms cultured in M7H9C pH 6.8.

Two-way Analysis of Variance

Two-way analysis of variance was conducted to compare twenty days and thirty days of incubation. Each treatment, i.e. pH, Oxyrase™ or hydrogen peroxide, was compared for twenty day and thirty days of incubation for each assay. Two-way ANOVA compared twenty days and thirty days but not each individual condition.

One-way Analysis of Variance

One-way analysis of variance was conducted to compare the different treatments at twenty or thirty days.



- A: Airtight container**
- B: 24 well microtiter plate**
- C: Cheese cloth soaked in Roccal**

Figure 3-1: Cut-away view of the culture chamber. 24 well microtiter plates allow for air circulation. The airtight container maintains humidity. Roccal provides moisture and limits fungal and microbial growth during culturing

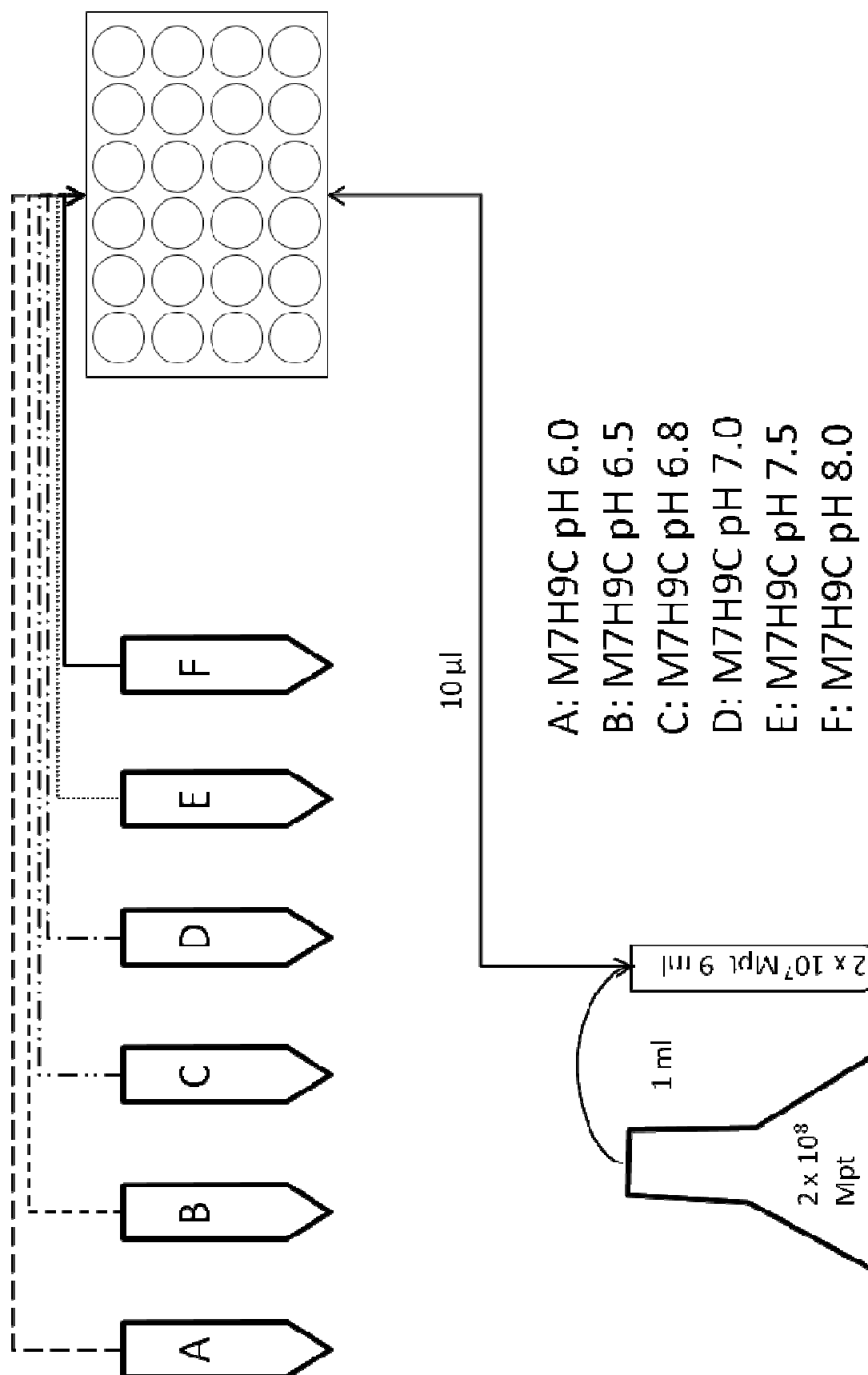


Figure 3-2: Dilution Diagram for Mpt biofilms cultured in M7H9C Δ pH

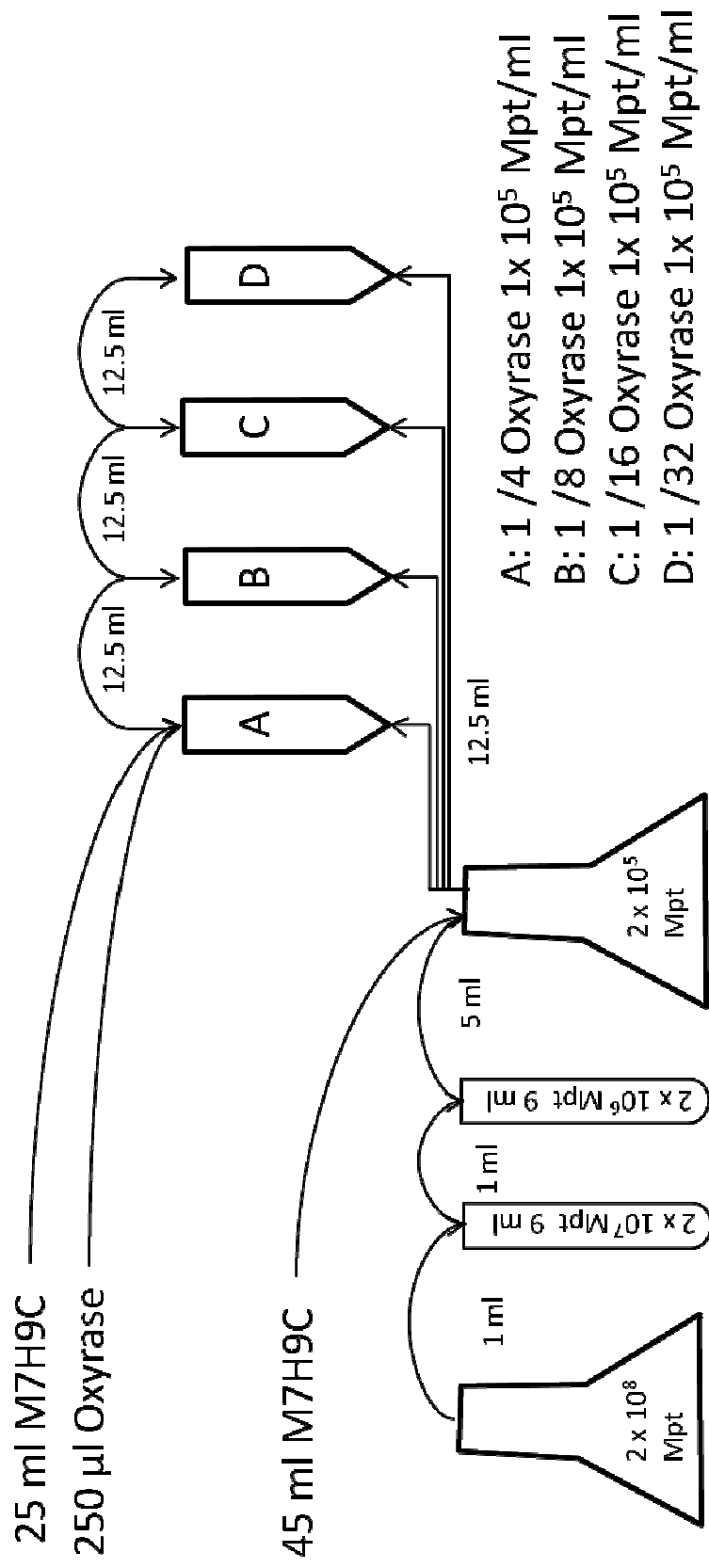


Figure 3-3: Dilution Diagram for Mpt biofilms cultured in M7H9C with Oxyrase

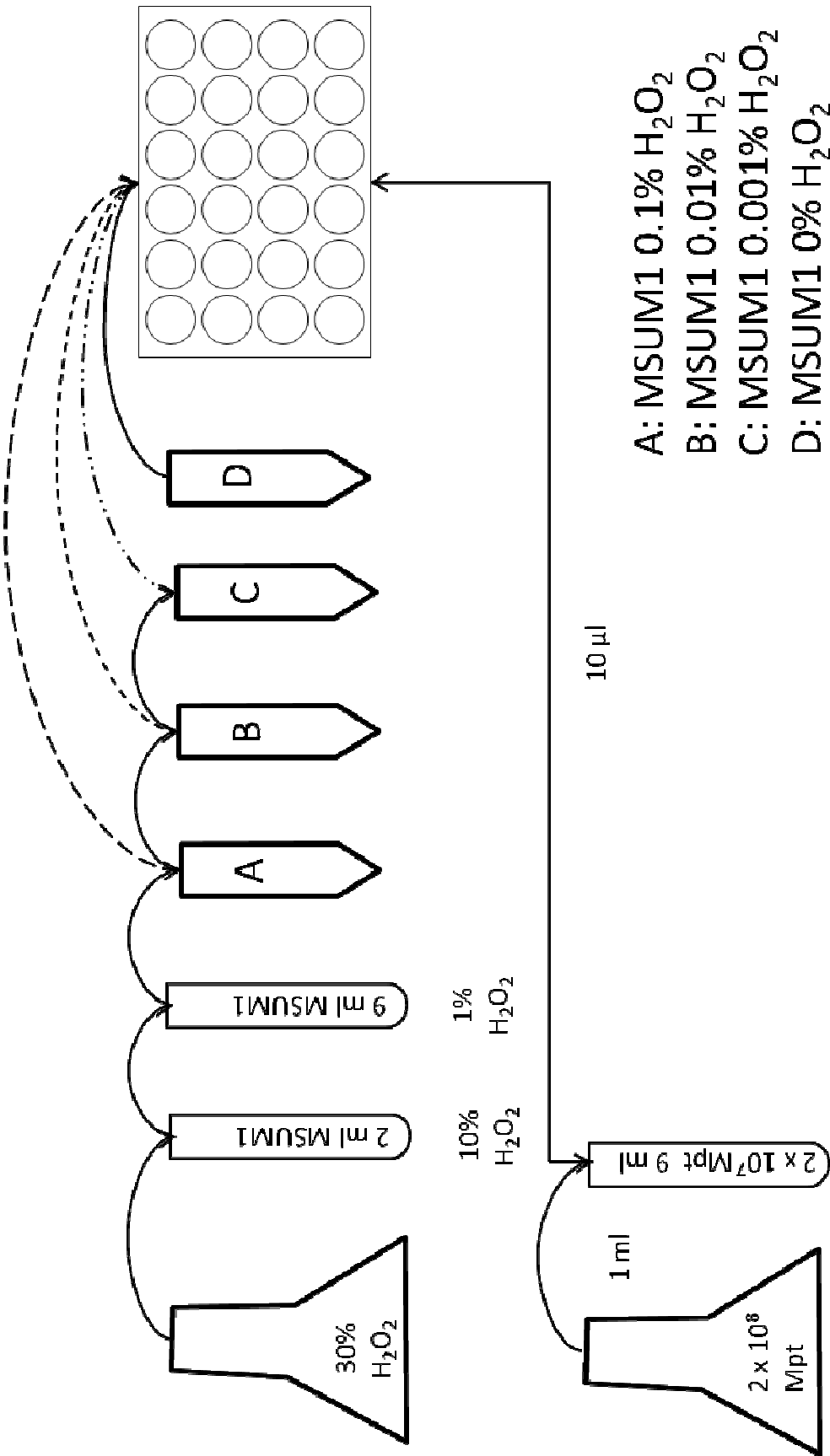


Figure 3-4: Dilution diagram for Mpt biofilms cultured in MSUM1 with hydrogen peroxide

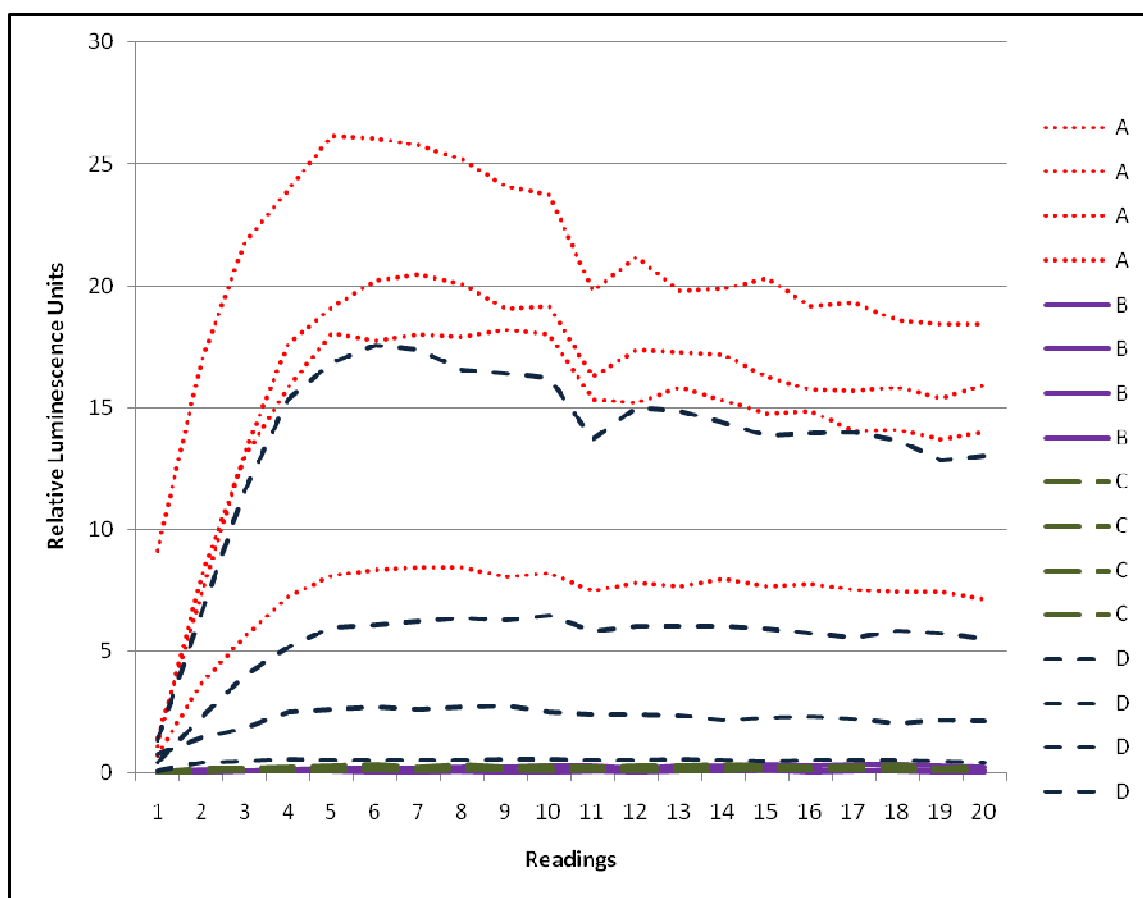


Figure 3-5: Change in luminescence over time.

A: Mpt in MSUM1 without H_2O_2 . B: Mpt in MSUM1 with 0.1% H_2O_2 .

C: Mpt with 0.01% H_2O_2 . D: Mpt with 0.001% H_2O_2 .

Chapter 4 Results

Section 1: Characterization of the effects of pH level on Mpt biofilms grown in M7H9C

Mycobacterium avium subspecies *paratuberculosis* PAMSUM 8 was cultured for twenty or thirty days in media adjusted to pH levels between pH 6.0 and pH 8.0. Adherent biofilms were extracted from growth plates and tested with one of three assays: Viability, ATP, or Biomass. The biofilm index combined data from the viability, cellularity, ATP and biomass assays to describe qualitatively which conditions allowed for the best biofilms.

Viability Ratio

Viability ratio was calculated by dividing the SYTO 9 fluorescence by the propidium iodide fluorescence for each replicate. The viability ratio represents the ratio of live cells to dead cells.

After twenty days (Figure 4-1, Panel A), the viability ratio was lower in biofilms initiated in alkaline conditions than biofilms grown in neutral or acidic conditions. The viability ratio was significantly lower in biofilms begun in pH 7.5 ($p < 0.05$) or pH 8.0 ($p < 0.05$) than biofilms grown in pH 6.8.

After thirty days (Figure 4-1, Panel B), the viability ratio was highest for biofilms initiated in pH 6.8, however this was not significant. As the pH level of the medium increased to pH 6.8 or decreased from pH 6.8, biofilms had lower viability ratios in the biofilm.

Two-way ANOVA indicated that there was no interaction between pH and incubation duration, however biofilm viability was significantly reduced from day twenty to day thirty ($p = 0.0008$). The effect of pH on biofilm viability was significant ($p = 0.0002$).

SYTO 9 Staining

SYTO 9 staining represents the amount of viable cells in a biofilm.

After twenty days of incubation, Mpt biofilms had similar SYTO 9 fluorescence in cultures beginning between pH 6.0 and pH 7.0 (Figure 4-2, Panel A) ($p > 0.05$). The amount of viable cells was significantly lower for Mpt initiated in alkaline conditions than in neutral or acidic conditions ($p < 0.05$).

After thirty days of incubation, biofilms had similar levels of fluorescence from SYTO 9 (Figure 4-2, Panel B) ($p > 0.05$). Biofilms begun in pH 6.8 had the most viable cells compared to biofilms begun in more acidic or more alkaline conditions; however, all treatments were statistically similar ($p > 0.05$). Despite the lack of statistical significance, there was an apparent trend showing the number of viable cells peaks in a narrow band of pH levels between pH 6.5 and 7.0 where Mpt biofilms had the most viable cells.

There was no interaction between pH and incubation duration indicated by two-way ANOVA. Time had insignificant effect on the amount of viable cells in biofilms ($p = 0.95$). There was significant effect from pH on the amount of viable cells ($p = 0.0001$).

Propidium Iodide Staining

Propidium iodide staining represents the amount of nonviable cells in a biofilm.

If propidium iodide only detects nonviable bacteria, biofilms initiated in alkaline conditions had significantly fewer nonviable cells after twenty days of incubation than biofilms initiated in media between pH 6.0 and 7.0 ($p < 0.05$) (Figure 4-3 Panel A). Biofilms started in media between pH 6.0 and 7.0 had similar fluorescence from propidium iodide ($p > 0.05$).

When incubated for thirty days (Figure 4-3, Panel B), an apparent trend showed the number of dead cells greatest at pH 6.8 and biofilms had fewer nonviable cells in higher or lower pH. All treatments were statistically similar to each other ($p > 0.05$). Despite the lack of statistical significance, at pH 6.0, pH 7.5 and pH 8, there were relatively few dead cells present in the biofilms.

As indicated by two-way ANOVA, there was no interaction between pH and incubation duration. The amount of nonviable cells slightly increased from day twenty to thirty ($p = 0.056$). The effect of pH on the amount of nonviable cells was significant ($p = 0.0002$).

Cellularity

Cellularity described the amount of cells, living and dead in biofilms. The relative fluorescence data for each replicate from propidium iodide were summed with the relative fluorescence data from SYTO 9.

Biofilms initiated in alkaline conditions had significantly fewer cells compared to biofilms incubated in pH 6.0, pH 6.5, pH 6.8 or pH 7.0 for twenty days ($p < 0.05$) (Figure 4-4, Panel A). The cellularity of biofilms incubated in pH 7.5 or pH 8.0 were statistically similar ($p > 0.05$). Initiated in conditions between pH 6.0 and pH 7.0, biofilms had similar amounts of cells ($p > 0.05$).

After thirty days, biofilms initiated in more acidic or more alkaline conditions than pH 6.8 had fewer cells in the biofilm than biofilms initiated in pH 6.8; however there was no statistical difference ($p > 0.05$). Biofilms started between pH 6.0 and pH 8.0 had statistically similar cellularity ($p > 0.05$) (Figure 4-4, Panel B).

Two-way ANOVA indicated that there was no interaction between pH and incubation duration. Time had negligible effect on cellularity ($p = 0.39$). Cellularity was significantly affected by pH ($p = 0.0001$).

ATP Assay

The ATP assay measured the amount of ATP present in biofilms and was therefore an indicator of metabolic activity. Biofilms with high amounts of ATP were regarded as more metabolically active than biofilms with low amounts of ATP.

After twenty days, biofilms initiated in alkaline conditions had negligible ATP (Figure 4-5, Panel A). Biofilms initiated between pH 6.0 and pH 7.0 had significantly higher amounts of ATP than biofilms started in pH 7.5 ($p < 0.05$) or pH 8.0 ($p < 0.05$). Biofilms initiated in conditions between pH 6.0 and pH 7.0 were statistically similar ($p > 0.05$).

Biofilms initiated in conditions more alkaline than pH 6.8 had less ATP after thirty days compared to biofilms initiated in other conditions ($p < 0.05$) (Figure 4-5, Panel B). Although not significant ($p > 0.05$) biofilms started at pH 6.8 had the most ATP. Biofilms grown in pH levels 6.0, 6.5, 6.8 or 7.0 had statistically similar levels of ATP ($p > 0.05$).

There was no interaction between pH and incubation duration according to two-way ANOVA. ATP content was not affected by incubation duration ($p = 0.77$). The amount of ATP content in biofilms was significantly affected by pH ($p < 0.0001$).

Biomass Assay

The biomass assay used crystal violet to quantify the amount of cells and extracellular material of biofilms.

The biomass assay resulted in similar results for biofilms incubated for twenty days regardless of the initial pH ($p > 0.05$) (Figure 4-6, Panel A). Visual examination showed negligible adherent cells and biofilm for replicates initiated in alkaline conditions. Biofilms initiated in acidic or neutral conditions were visually more robust than biofilms initiated in alkaline conditions.

After thirty days biofilms initiated at pH 8.0 had significantly more biomass than biofilms incubated in pH 6.0 ($p < 0.01$), pH 6.5 ($p < 0.01$) or pH 6.8 ($p < 0.01$) (Figure 4-6, Panel B). There was an apparent trend that there was more biomass as the pH level of the medium became more alkaline. When visually inspected, there were inconsequential amounts of biofilm adhering to the substrates for cells incubated in alkaline conditions. Visually, biofilms begun in conditions more acidic than pH 7.5 were more robust than biofilms cultured in alkaline conditions.

Two-way ANOVA indicated that there was no interaction between pH and incubation duration. The effect of time on biomass was significant ($p < 0.0001$). The effect of pH on the amount of nonviable cells was significant ($p = 0.0001$).

Biofilm index

The biofilm index was developed to compare all of the treatments using all assays to determine the best conditions for biofilm formation. The data for the biofilm index was derived by normalizing the data from the viability ratio, cellularity, ATP assay and biomass assay allowed each assay to have equal weight.

After twenty days this index indicated that biofilms were similar among different treatments (Figure 4-7, Panel A). There was an apparent trend that biofilms were the most robust in pH 6.8 conditions and were less robust in more acidic or more alkaline conditions.

Biofilms were most robust in pH 7.0 or pH 8.0 conditions after thirty days (Figure 4-7, Panel B). In pH 7.5 conditions, biofilms scored lower than pH 7.0 or pH 8.0 conditions. The mean score for treatments more acidic than pH 6.8 or pH 7.5 were less than the scores for pH 7.0 or pH 8.0.

Section 2: Characterization of the effects of oxygen deprivation on Mpt biofilms

Mycobacterium avium subspecies *paratuberculosis* PAMSUM 8 was cultured for twenty or thirty days using Oxyrase™ to depleted oxygen content of the media. A control group was cultured without hypoxic conditions. Adherent biofilms were extracted from growth plates then measured using three assays: viability, ATP or biomass. The biofilm index combines data from the viability, cellularity, ATP and biomass assays to describe which condition produced the best biofilms.

The effects of Oxyrase™ on oxygen levels over time in an open system

In closed systems Oxyrase™ reduces the oxygen levels in media and maintains anaerobic conditions indefinitely. In open systems oxygen dissolves into the medium and does not remain hypoxic indefinitely. Using an YSI 5300 Biological Oxygen Meter (YSI inc. Yellow Springs, Ohio), the change in oxygen levels over time were studied (Figure 4-8). The oxygen levels of PBS treated with 1/8 Oxyrase™ dropped quickly and remained low for about 24 hours before slowly returning to normal. The oxygen levels of PBS treated with 1/16 Oxyrase™ dropped quickly as well, however the oxygen levels returned to normal quicker and did not become as hypoxic. This indicated that hypoxic conditions were present for only a short period of time during the incubation of cells.

Viability ratio

The viability ratio represents the ratio of live cells to dead cells. The viability ratio was calculated by dividing the SYTO 9 fluorescence by the propidium iodide fluorescence for each replicate.

When exposed to Oxyrase™ and incubated for twenty days, Mpt had lower viability ratios than when not exposed to Oxyrase™ ($p < 0.01$) (Figure 4-9, Panel A). The viability ratios were not significantly different among the different concentrations of Oxyrase™ ($p > 0.05$).

When exposed to Oxyrase™ and cultured for thirty days, Mpt had lower viability ratios than when not exposed to Oxyrase™ ($p < 0.001$) (Figure 4-10, Panel B). The viability ratios were similar among the different concentrations of Oxyrase™ ($p > 0.05$).

Two-way ANOVA indicated that there was slight interaction between Oxyrase™ and incubation duration ($p = 0.087$). Incubation duration affected the viability of biofilms. Biofilms had lower viability at day thirty than at day twenty ($p = 0.03$). Oxyrase™ had a negative effect on biofilm viability ($p < 0.0001$).

SYTO 9 staining

SYTO 9 staining represents the amount of viable cells in a biofilm.

When exposed to hypoxic conditions during twenty days of incubation, Mpt biofilms had fewer viable cells present than Mpt biofilms not exposed to hypoxic conditions ($p < 0.001$) (Figure 4-10 Panel A). Biofilms exposed to hypoxic conditions had statistically similar amounts of viable cells ($p > 0.05$).

When cultured without Oxyrase™, Mpt biofilms had significantly more viable cells after thirty days than Mpt biofilms cultured with Oxyrase™ ($p < 0.001$) (Figure 4-10, Panel B). Mpt exposed to hypoxic conditions had similar quantities of viable cells ($p > 0.05$).

There was no interaction between incubation time and Oxyrase™ concentration for the amount of viable cells in a biofilm ($p = 0.89$). Incubation duration had insignificant effect on the amount of viable cells in a biofilm ($p = 0.56$). There was significant negative impact from Oxyrase™ on viable cells in a biofilm ($p < 0.0001$).

Propidium Iodide staining

Propidium iodide represents the amount of nonviable cells in a biofilm.

When exposed to hypoxic conditions during twenty days of incubation, Mpt biofilms had fewer dead cells present than Mpt not exposed to hypoxic conditions ($p < 0.001$) (Figure 4-11, Panel A). Biofilms exposed to hypoxic conditions had similar amounts of dead cells ($p > 0.05$).

After thirty days, when exposed to hypoxic conditions, Mpt biofilms had fewer dead cells than when not exposed to hypoxic conditions ($p < 0.001$) (Figure 4-11, Panel B). Biofilms exposed to hypoxic conditions had similar quantities of nonviable cells ($p > 0.05$).

Two way ANOVA indicated that there was no interaction between incubation time and Oxyrase™ concentration for the amount of nonviable cells in a biofilm ($p = 0.16$). The amount of nonviable cells was significantly reduced due to incubation time ($p = 0.039$). There were significantly fewer nonviable cells when cultures were incubated with Oxyrase™ ($p < 0.0001$).

Cellularity

The relative fluorescence data for each replicate from propidium iodide were summed with the relative fluorescence data from SYTO 9. Cellularity described the amount of cells, living and dead in biofilms.

When exposed to hypoxic conditions during twenty days of incubation, Mpt biofilms had fewer cells present than Mpt biofilms not exposed to hypoxic conditions ($p < 0.001$) (Figure 4-12, Panel A). Biofilms exposed to hypoxic conditions had similar amounts of cells present in the biofilms ($p > 0.05$).

There were significantly fewer cells present after thirty days when Mpt was exposed to hypoxic conditions than when not exposed to hypoxic conditions ($p < 0.001$) (Figure 4-12, Panel B). When cultured for thirty days with Oxyrase™, biofilms had similar cellularity ($p > 0.05$).

Oxyrase™ and incubation time had no interaction on the cellularity of biofilms ($p = 0.45$). There was insignificant effects from incubation duration on the cellularity of biofilms ($p = 0.16$). Oxyrase™ concentration significantly reduced the cellularity of biofilms ($p < 0.0001$).

ATP Assay

When exposed to hypoxic conditions during twenty days of incubation, Mpt biofilms had negligible ATP present. All biofilms cultured with Oxyrase™ had similar ATP content ($p > 0.05$) (Figure 4-13, Panel A). Biofilms that were not exposed to hypoxic conditions during twenty days of incubation had significantly more ATP than biofilms exposed to hypoxic conditions ($p < 0.001$).

Exposure to Oxyrase™ during thirty days of incubation resulted in limited amounts of ATP present when compared to Mpt incubated without Oxyrase™ ($p < 0.001$). Cells incubated with 1/4, 1/8, 1/16 or 1/32 Oxyrase™ had similar amounts of ATP present ($p > 0.05$).

There was no interaction between Oxyrase™ and incubation time on the ATP content in biofilms ($p = 0.80$). Time had insignificant influence on ATP content ($p = 0.31$). ATP concentration was significantly reduced by the presence of Oxyrase™ ($p < 0.0001$).

Biomass Assay

The biomass assay used crystal violet to quantify the amount of cells and extracellular material of biofilms.

When exposed to hypoxic conditions during twenty days of incubation, Mpt biofilms had negligible biomass (Figure 4-14 Panel A). Biofilms not exposed to hypoxic conditions had significantly more biomass than biofilms exposed to hypoxic conditions ($p < 0.01$). Biofilms exposed to hypoxic conditions had similar amounts of biomass and were not significantly different than each other ($p > 0.05$).

After thirty days incubation with Oxyrase™, biofilms had higher biomass than Mpt incubated without Oxyrase™ (Figure 4-14 A). Mpt cultured in the absence of Oxyrase™ had significantly less biomass than Mpt cultured with Oxyrase™ ($p < 0.001$). Mpt cells incubated with 1/4, 1/8, 1/16 or 1/32 Oxyrase™ had similar amounts of biomass ($p > 0.05$). Visually biofilms were more robust in the absence of Oxyrase™ than in the presence of Oxyrase™.

There was significant interaction between Oxyrase™ and incubation duration on the biomass of biofilms ($p < 0.0001$). Oxyrase™ had negligible effects on biofilm biomass ($p = 0.85$). Time had insignificant effects on the biomass of biofilms ($p = 0.15$).

Biofilm Index

The biofilm index was developed to compare all of the treatments using all assays to determine the best conditions for biofilm formation. The data for the biofilm index was derived by normalizing the data from the viability ratio, cellularity, ATP and biomass assay allowed each assay to have equal weight.

Biofilms not exposed to hypoxic conditions had a higher viability ratio, more ATP and more biomass than biofilms exposed to hypoxic conditions during twenty days of incubation (Figure 4-14, Panel A). When exposed to hypoxic conditions, Mpt biofilms had low viability, few cells, negligible ATP and little biomass. Biofilms exposed to 1/4, 1/8, 1/16 or 1/32 Oxyrase™ had similar viability, cellularity, ATP and biomass.

After thirty days of incubation with or without Oxyrase™, biofilms had similar biofilm scores (Figure 4-14, Panel B). Mpt cultured with Oxyrase™ had lower viability, cellularity but had higher biomass than Mpt cultured without Oxyrase™.

Section 3: Characterization of the effects of oxidative stress on biofilms of Mpt

Mycobacterium avium subspecies *paratuberculosis* PAMSUM 8 was cultured for twenty or thirty in the absence of H₂O₂ or in the presence of 0.1% H₂O₂, 0.01% H₂O₂ or 0.001% H₂O₂. Biofilms adhering to coverslips were extracted from growth plates and measured using three assays: viability, ATP or biomass. The biofilm index combined data from the viability, cellularity, ATP and biomass assays to describe which condition produced the best biofilms.

Viability Ratio

The viability ratio represents the ratio of live cells to dead cells. The viability ratio was calculated by dividing the SYTO 9 fluorescence by the propidium iodide fluorescence for each replicate.

After twenty days of incubation with or without hydrogen peroxide, all of the treatments had similar viability ratios ($p > 0.05$) (Figure 4-16, Panel A). When incubated in the presence of 0.001% H₂O₂, Mpt biofilms had slightly more viable cells than Mpt incubated in the absence of H₂O₂ ($p > 0.05$).

After thirty days, the viability ratios of biofilms initiated in the presence of 0.1% H₂O₂ or 0.01% H₂O₂ were significantly lower than the viability ratios of biofilms initiated in the absence of H₂O₂ ($p < 0.05$) (Figure 4-16, Panel B). Biofilms started in 0.001% H₂O₂ had significantly higher

viability than biofilms started in 0.01% H_2O_2 or 0.1% H_2O_2 . The viability of Mpt biofilms incubated in 0.001% H_2O_2 was similar to Mpt biofilms incubated without H_2O_2 .

There was no interaction between H_2O_2 concentration and incubation duration on viability of biofilms ($p = 0.43$). Incubation time significantly reduced the viability of biofilms ($p = 0.0002$). Concentration of H_2O_2 had insignificant effects on biofilm viability ($p = 0.12$).

SYTO 9 Staining

SYTO 9 staining represents the amount of viable cells in a biofilm.

When incubated with or without H_2O_2 for twenty days, Mpt biofilms had similar SYTO 9 fluorescence ($p > 0.05$) (Figure 4-17, Panel A). Although not statistically significant there was an apparent trend with decreased SYTO 9 staining with increased H_2O_2 concentration.

When incubated for thirty days, there was a significantly more SYTO 9 fluorescence from biofilms grown in the absence of H_2O_2 than biofilms grown in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.01$) (Figure 4-17, Panel B). There was no significant difference among treatments incubated in the presence of H_2O_2 for thirty days ($p > 0.05$). Although not significant there was an apparent trend indicating that as H_2O_2 concentration increased, the amount of viable cells decreased.

There was insignificant interaction between H_2O_2 and incubation time on the amount of viable cells ($p = 0.61$). Concentration of H_2O_2 negatively influenced the amount of viable cells in biofilms ($p = 0.0014$). Incubation time reduced the amount of viable cells in biofilms ($p = 0.0036$).

Propidium Iodide Staining

Propidium iodide staining represents the amount of nonviable cells in a biofilm.

After twenty days, Mpt incubated in the absence of H_2O_2 had more cells stained with propidium iodide than Mpt incubated in the presence of H_2O_2 (Figure 4-18, Panel A). Biofilms grown in the absence of H_2O_2 had significantly higher fluorescence from propidium iodide than biofilms grown in the presence of H_2O_2 ($p < 0.01$). Biofilms grown in the presence of H_2O_2 had similar levels of fluorescence from propidium iodide ($p > 0.05$).

After thirty days, Mpt biofilms initiated in the absence of H_2O_2 had similar relative fluorescence signals from propidium iodide to that of biofilms initiated in the presence of 0.001% H_2O_2 ($p > 0.05$) (Figure 4-18, Panel B). Biofilms initiated in the absence of H_2O_2 had a significantly higher relative fluorescence signals from propidium iodide than biofilms initiated in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.01$). Biofilms with initial H_2O_2 concentrations of 0.1%, 0.01% or 0.001% H_2O_2 were statistically similar to each other ($p > 0.05$).

Hydrogen peroxide significantly diminished the amount of nonviable cells in Mpt biofilms ($p < 0.0001$). Time had insignificant effect on the quantity of dead cells in biofilms ($p = 0.12$). There was no interaction between H_2O_2 concentration and time ($p = 0.89$).

Cellularity

Cellularity described the amount of cells, living and dead in biofilms. The relative fluorescence data from propidium iodide were summed with the relative fluorescence units from SYTO 9 for each replicate.

After twenty days, biofilms incubated without H_2O_2 had greater cellularity than biofilms incubated with 0.01% H_2O_2 ($p < 0.05$) (Figure 4-19, Panel A). Biofilms initiated in the absence of H_2O_2 had similar cellularity to biofilms initiated in 0.001% or 0.1% H_2O_2 ($p > 0.05$). Cultures initiated with concentrations of 0.001% H_2O_2 , 0.01% H_2O_2 or 0.1% H_2O_2 had similar cellularity ($p > 0.05$). There was an apparent trend showing that as the amount of H_2O_2 increased, the amount of cells present decreased.

Biofilms incubated in the absence of H_2O_2 for thirty days had higher cellularity than biofilms grown in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.01$) (Figure 4-19, Panel B). Cells incubated in the absence of H_2O_2 appeared to have slightly higher cellularity than biofilms incubated with 0.001% H_2O_2 but this was not significant ($p > 0.05$). The cellularity of biofilms initiated in the presence of 0.001% H_2O_2 , 0.01% H_2O_2 , or 0.1% H_2O_2 were similar ($p > 0.05$). There was an apparent trend that as H_2O_2 concentration increased that the amount of cells decreased.

Cellularity was significantly reduced by the concentration of H_2O_2 ($p < 0.0001$). Duration of incubation negatively affected cellularity of Mpt biofilms ($p = 0.0074$). There was no interaction between time and H_2O_2 concentration on cellularity ($p = 0.81$).

ATP Assay

The ATP assay measured the amount of ATP present in biofilms and was therefore an indicator of metabolic activity. Biofilms with high amounts of ATP were regarded as more metabolically active than biofilms with low amounts of ATP.

After twenty days, the amount of ATP in Mpt biofilms grown in the absence of H_2O_2 was significantly higher than biofilms initiated in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.001$), both of which had negligible amounts of ATP (Figure 4-20, Panel A). While the mean luminescence value for biofilms grown in the presence of 0.001% H_2O_2 was lower than without H_2O_2 , the difference was not significant ($p > 0.05$). Biofilms grown in the presence of 0.001% H_2O_2 had higher amounts of ATP than biofilms grown in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.05$).

The amount of ATP in Mpt biofilms grown for thirty days in the absence of H_2O_2 was greater than that for biofilms initiated in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.001$) (Figure 4-20, Panel B). Biofilms incubated in the presence of 0.001% H_2O_2 or without H_2O_2 were statistically similar ($p > 0.05$). After thirty days in the presence of 0.001% H_2O_2 biofilms had significantly higher amounts of ATP than biofilms grown in the presence of 0.1% or 0.01% H_2O_2 ($p < 0.05$).

The amount of ATP was significantly reduced by the concentration of H_2O_2 ($p < 0.0001$). Time had no effect on the concentration of ATP ($p = 0.48$). There was no interaction between H_2O_2 concentration and time ($p = 0.89$).

Biomass Assay

The biomass assay used crystal violet to quantify the amount of cells and extracellular material of biofilms.

After twenty days, the amount of biomass of biofilms grown with or without hydrogen peroxide was statistically similar between treatments ($p > 0.05$) (Figure 4-21, Panel A).

The thirty day old Mpt biofilms showed a similar pattern of optical densities as the twenty day old Mpt biofilms (Figure 4-21, Panel B). None of the treatments were significantly different from each other ($p > 0.05$).

Biofilm Index

The biofilm index was developed to compare all of the treatments using all assays to determine the best conditions for biofilm formation. The data for the biofilm index was derived by normalizing data from the viability ratio, cellularity, ATP and biomass assays using Mpt cultured without H_2O_2 as the control treatment. Normalizing allowed each assay to have equal weight.

After twenty days, Mpt initiated in the absence of H_2O_2 formed better biofilms than biofilms initiated in the presence of H_2O_2 (Figure 4-22, Panel A). Mpt initiated with H_2O_2 were similar to each other, but cells initiated in the presence of 0.001% H_2O_2 formed slightly better biofilms than cells initiated in the presence of 0.01% H_2O_2 or 0.1% H_2O_2 .

The best biofilms after thirty days were those not exposed to H_2O_2 (Figure 4-22, Panel B). Mpt initiated in the absence of H_2O_2 had slightly better biofilms than those initiated in 0.001% H_2O_2 . Mpt initiated in the absence of H_2O_2 formed better biofilms than those initiated in the presence of 0.01% H_2O_2 or 0.1% H_2O_2 . Biofilms initiated in the presence of 0.001% H_2O_2 were not different than biofilms initiated in 0.1% H_2O_2 .

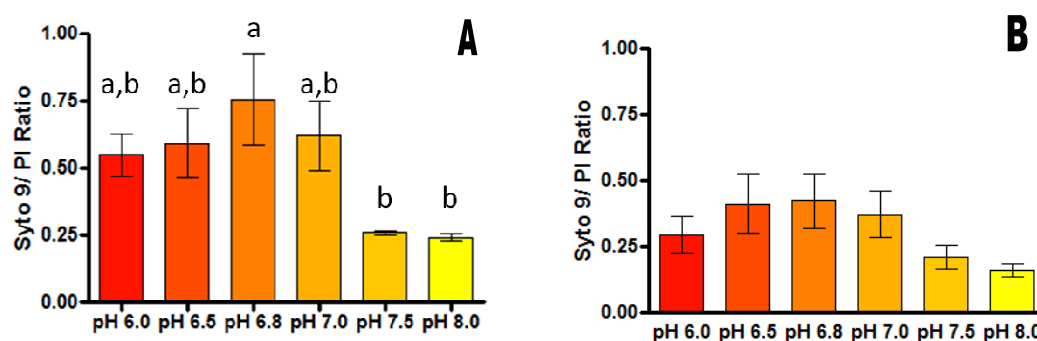


Figure 4-1: Alkaline conditions inhibited the viability of Mpt in biofilms. After incubation in media at various pH levels, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). Viability ratio is the ratio of SYTO 9 fluorescence to propidium iodide fluorescence. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

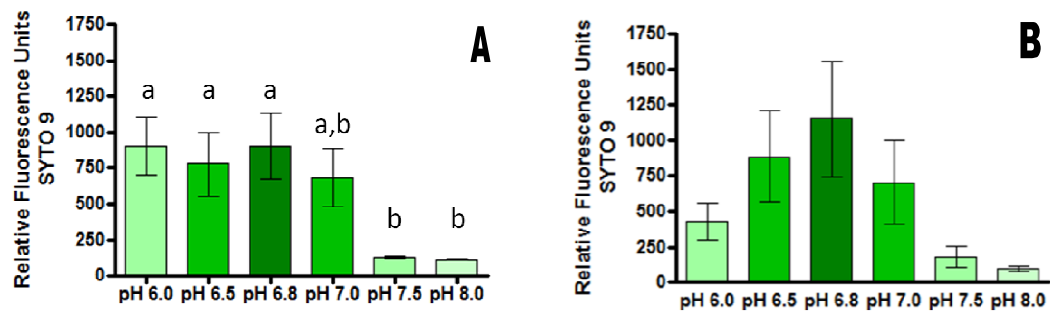


Figure 4-2: Alkaline conditions limited the amount of viable Mpt cells in biofilms. After incubation in media at various pH levels for twenty or thirty days, Mpt biofilms were stained with SYTO 9 and propidium iodide. SYTO 9 staining represents the amount of viable cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

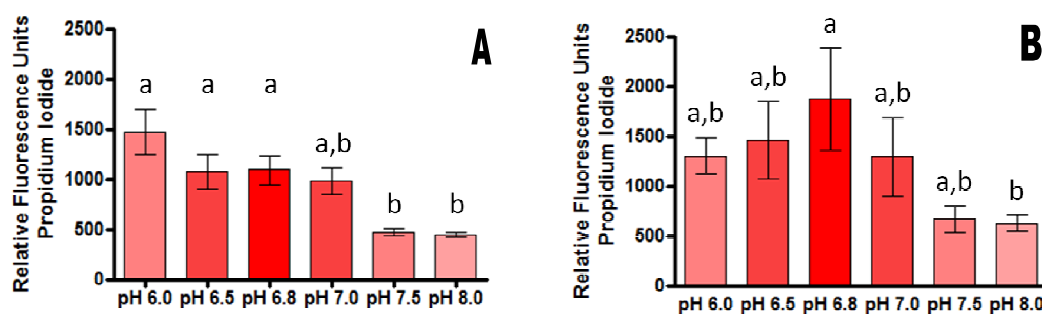


Figure 4-3: Alkaline conditions limited the amount of nonviable Mpt cells in biofilms.

After incubation in media at various pH levels for twenty or thirty days, Mpt biofilms were stained with SYTO 9 and propidium iodide. Propidium iodide staining describes how many cells died over the course of the incubation period. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

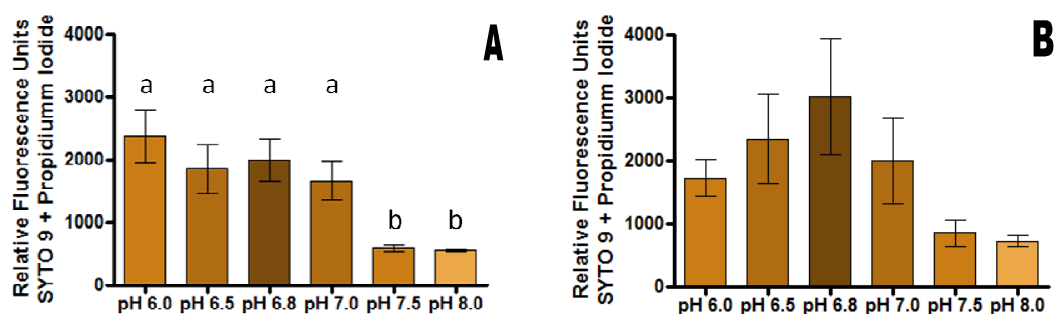


Figure 4-4: Alkaline conditions limited the cellularity of Mpt biofilms. After incubation in media at various pH levels for twenty or thirty days, Mpt biofilms were stained with SYTO 9 and propidium iodide, then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). The fluorescence of SYTO 9 and propidium iodide of biofilms represents the cellularity or amount of cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

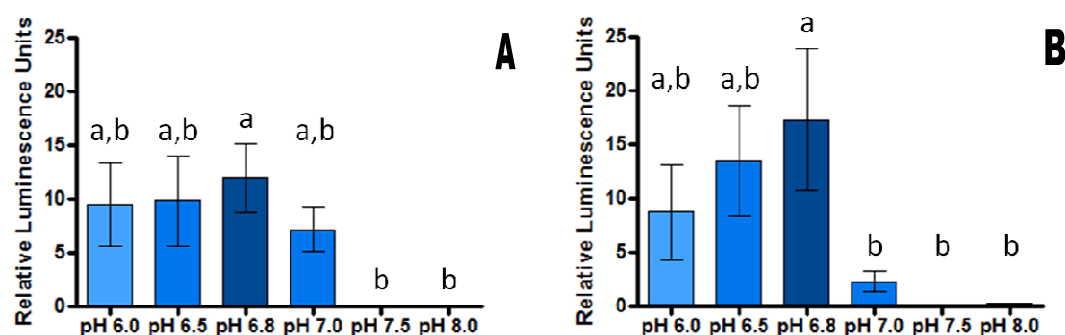


Figure 4-5: Alkaline conditions limited the amount of ATP present in Mpt biofilms.

After incubation in media at various pH levels for twenty or thirty days, Mpt cells in biofilms were lysed and the amount of ATP was measured using a bioluminescence reaction and were read in a Fluoroskan FL luminometer. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

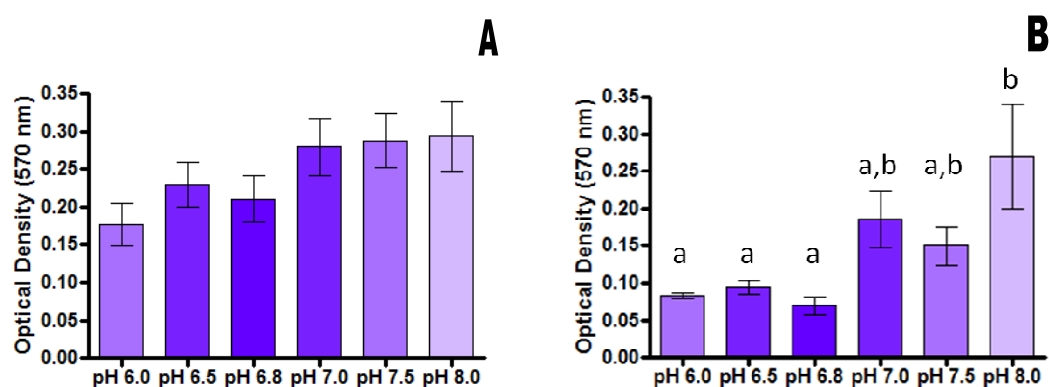


Figure 4-6 Alkalinity caused an apparent increase in biomass. After incubation in media at various pH levels for twenty or thirty days, Mpt biofilms were stained with crystal violet then washed with deionized water. Crystal violet was solubilized with 95% ethanol, and then the crystal violet-ethanol solution was measured for optical density at 570 nm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.01$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

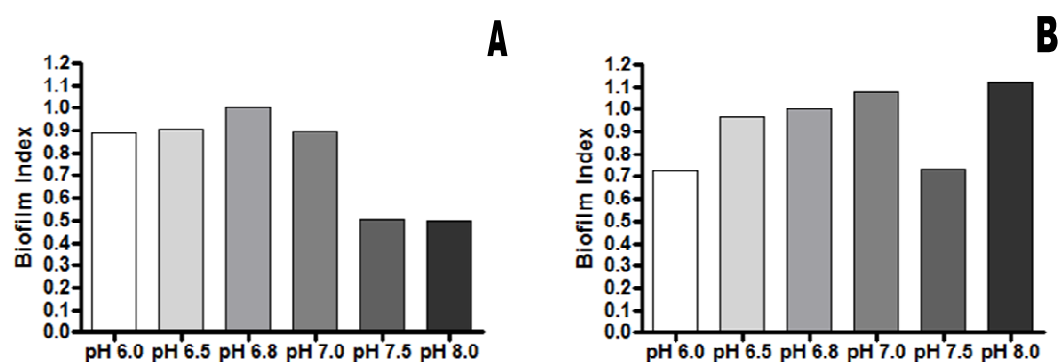


Figure 4-7: Alkaline conditions inhibit biofilm development. The biofilm index combines normalized data from viability ratio, cellularity, ATP and biomass assays. Data were normalized so that the mean for pH 6.8 equals 1. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days.

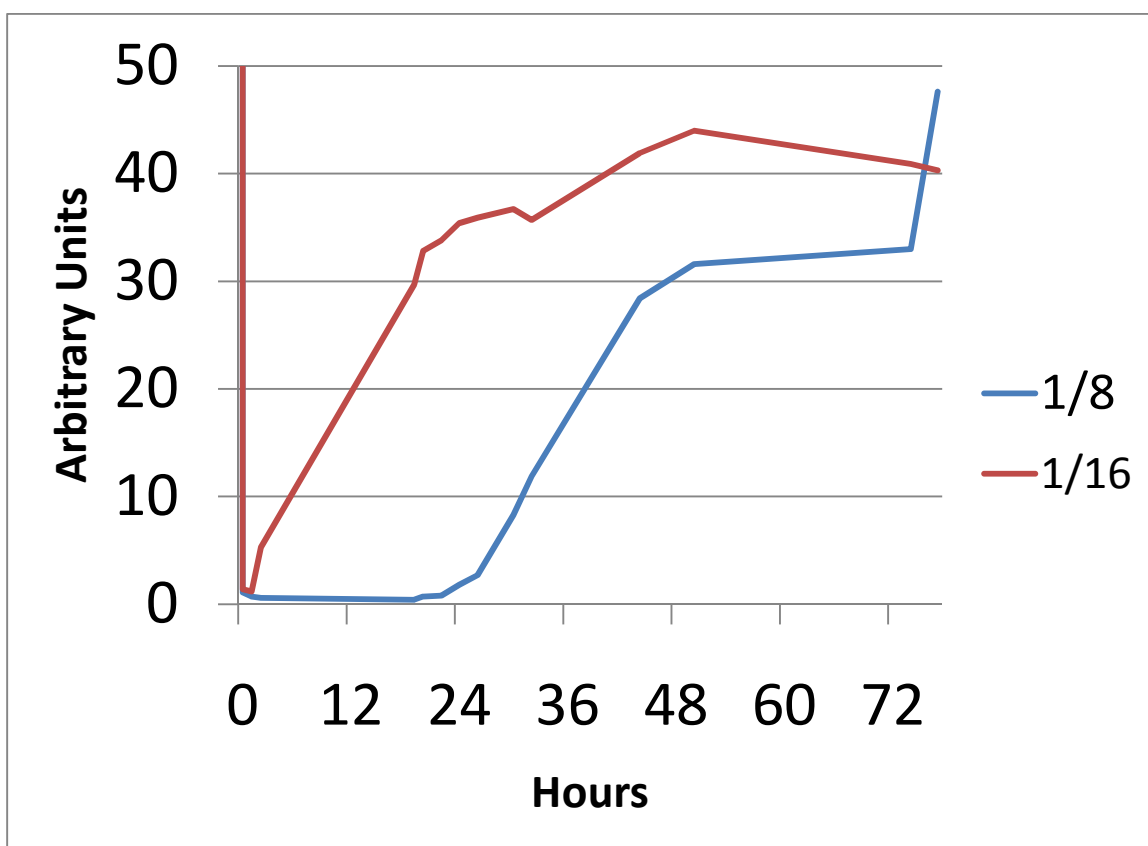


Figure 4-8: Oxyrase™ reduces the oxygen concentration but cannot maintain hypoxic conditions indefinitely. Oxyrase™ at 1/8 of the recommended dose maintained low oxygen levels longer than Oxyrase™ at 1/16 of the recommended dose.

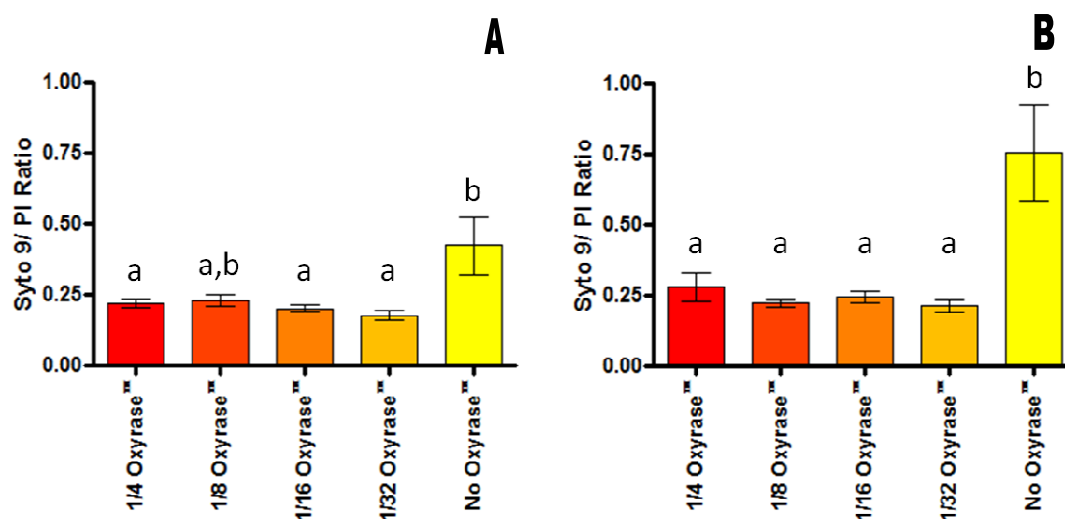


Figure 4-9: Hypoxic conditions limited viability of Mpt when cultured with Oxyrase™.

After incubation in media treated with various concentrations of Oxyrase™, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). Viability ratio is the ratio of SYTO 9 fluorescence to propidium iodide fluorescence. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.01$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

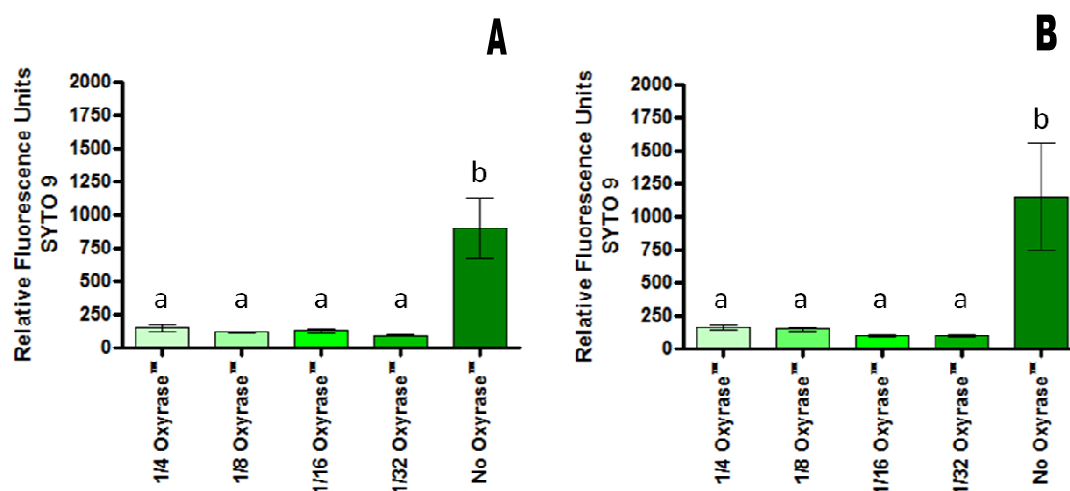


Figure 4-10: Hypoxic conditions limited the amount of viable Mpt when cultured with Oxyrase™. After incubation in media treated with various concentrations of Oxyrase™, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluormeter with excitation wavelength of 485 nm, emission wave length of 538 nm (green). SYTO 9 staining represents the amount of viable cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.001$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

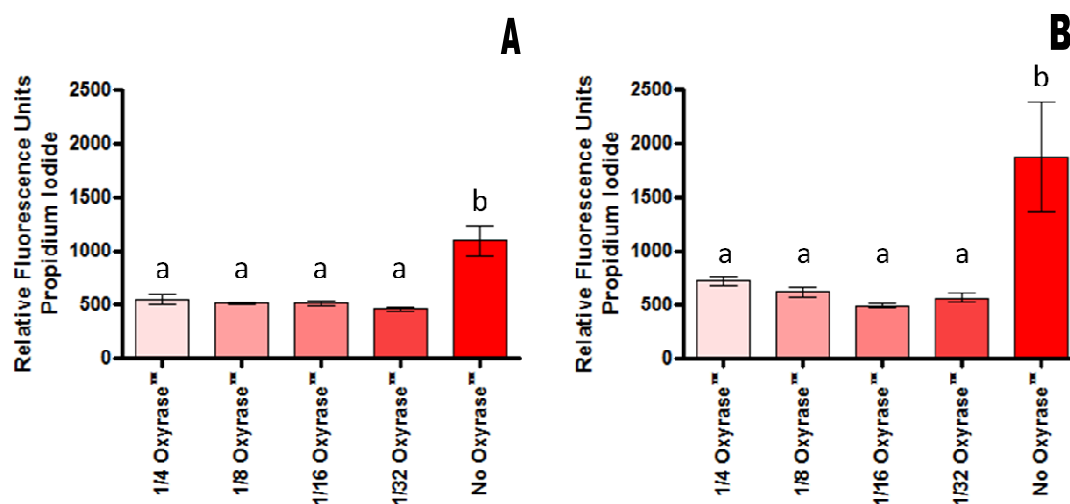


Figure 4-11: Hypoxic conditions limited the amount of nonviable Mpt when cultured with Oxyrase™. After incubation in media treated with various concentrations of Oxyrase™, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave length of 635 nm (red). Propidium iodide staining describes how many cells died over the course of the incubation period. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.001$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

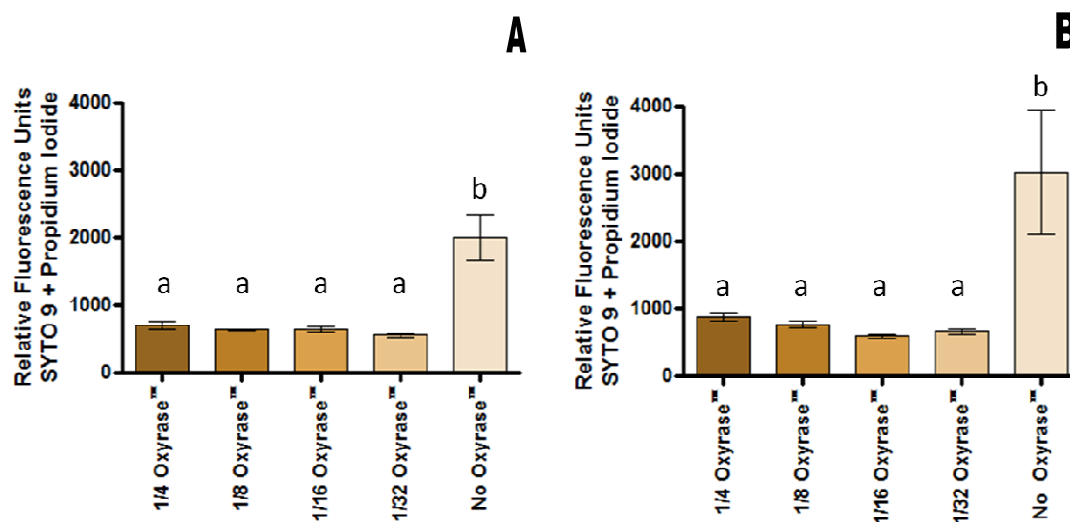


Figure 4-12: Hypoxic conditions limited cellularity of Mpt when cultured with Oxyrase™. After incubation in media treated with various concentrations of Oxyrase™, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). The fluorescence of SYTO 9 and propidium iodide of biofilms represents the cellularity or amount of cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.001$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

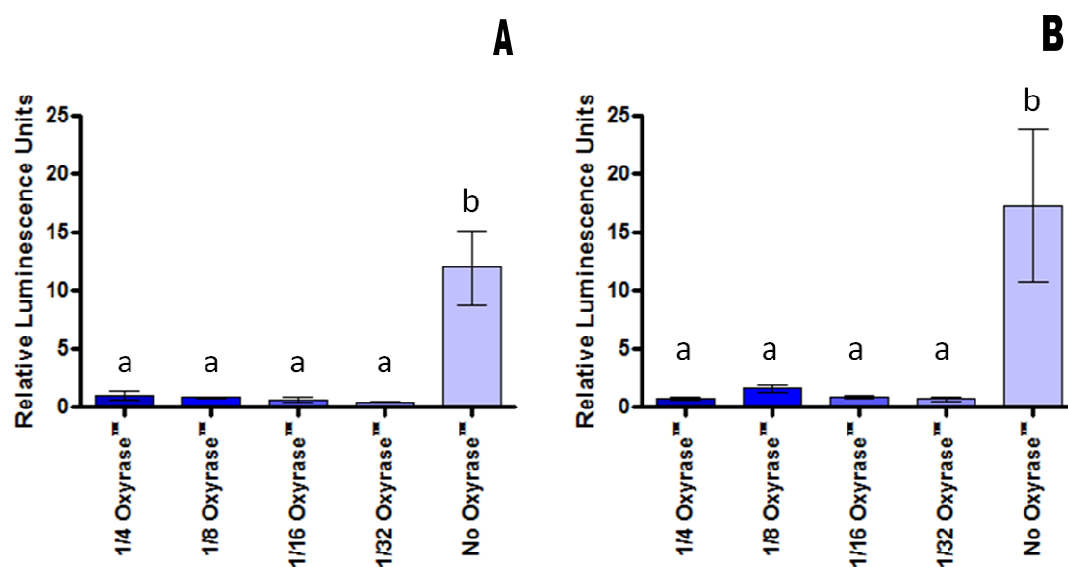


Figure 4-13: Hypoxic conditions inhibited metabolic activity of Mpt when cultured with Oxyrase™. After incubation in media treated with various concentrations of Oxyrase™, for twenty or thirty days, Mpt cells in biofilms were lysed and the amount of ATP was measured using a bioluminescence reaction and were read in a Fluoroskan FL luminometer. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.001$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

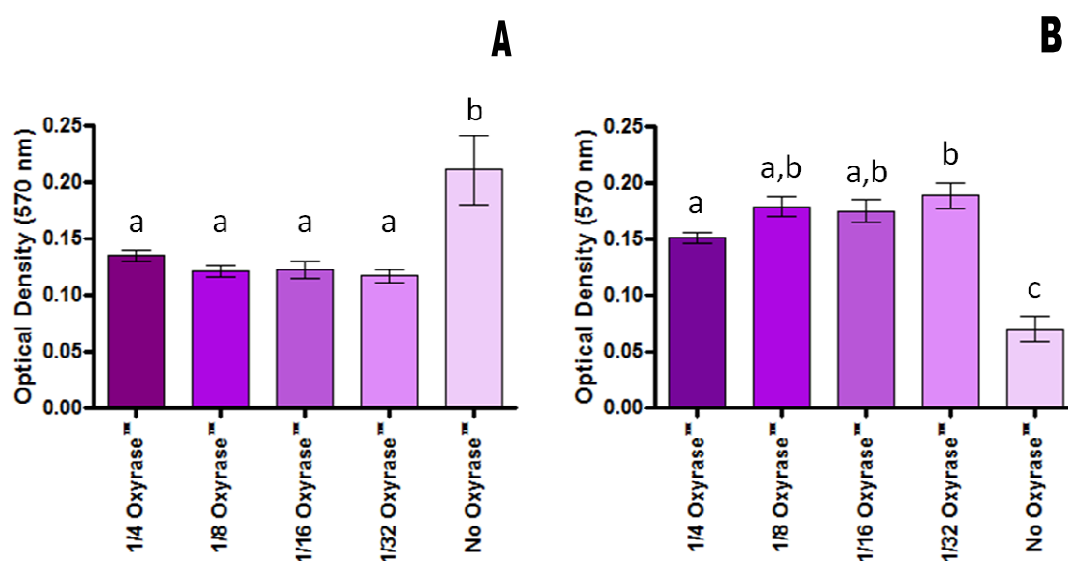


Figure 4-14: Hypoxic conditions resulted in an apparent increase in biomass of Mpt biofilms when cultured with Oxyrase™. After incubation in media treated with various concentrations of Oxyrase™, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.01$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

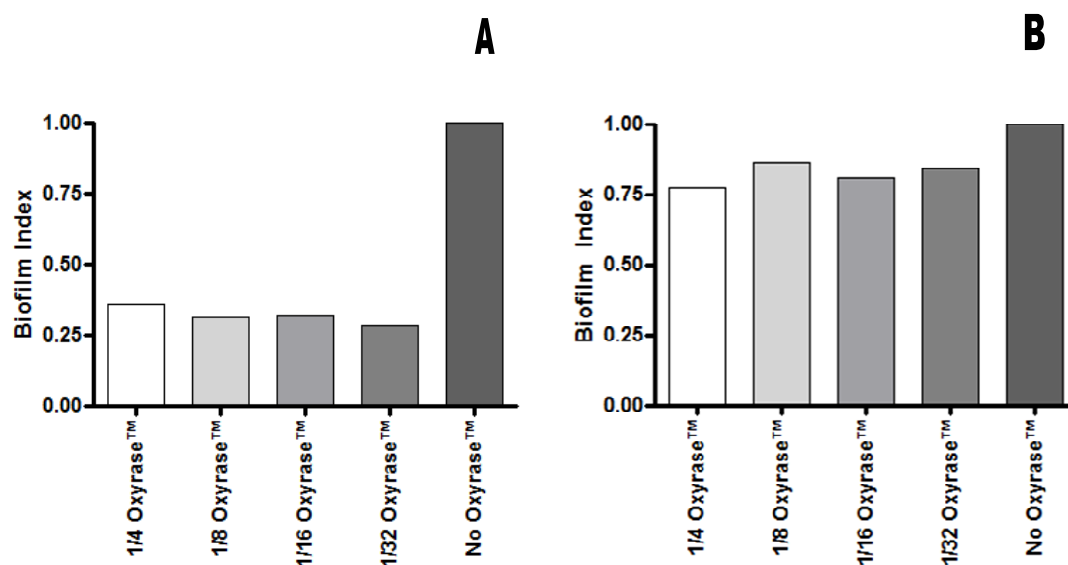


Figure 4-15: Hypoxic conditions inhibited growth of Mpt when cultured with Oxyrase™ for 20 days. The biofilm index combines normalized data from viability ratio, cellularity, ATP and biomass assays. Data were normalized so that the mean for pH 6.8 equals 1. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days.

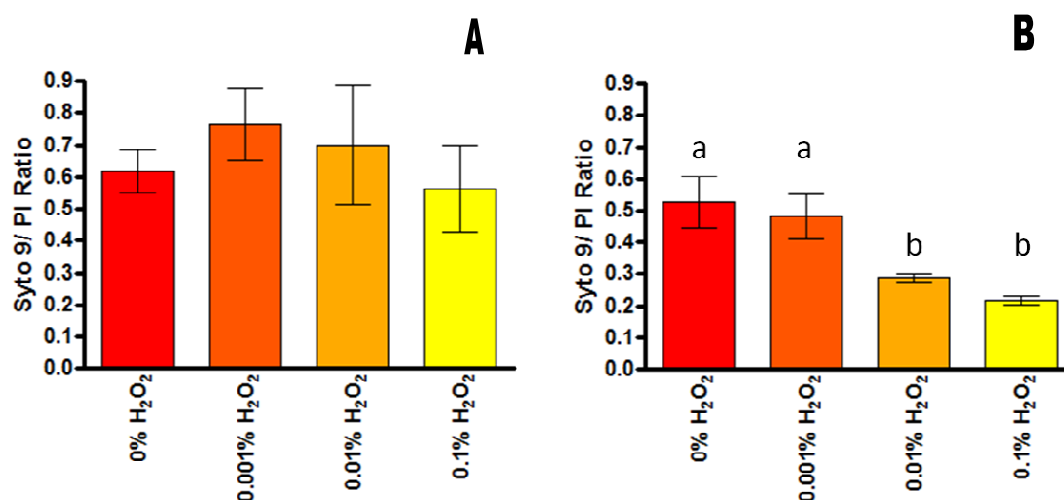


Figure 4-16: Hydrogen peroxide diminished the viability of Mpt. After twenty or thirty days of incubation in the presence of hydrogen peroxide, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). Viability ratio is the ratio of SYTO 9 fluorescence to propidium iodide fluorescence. Panel A represents biofilms initiated for twenty days. Panel B represents biofilms initiated for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

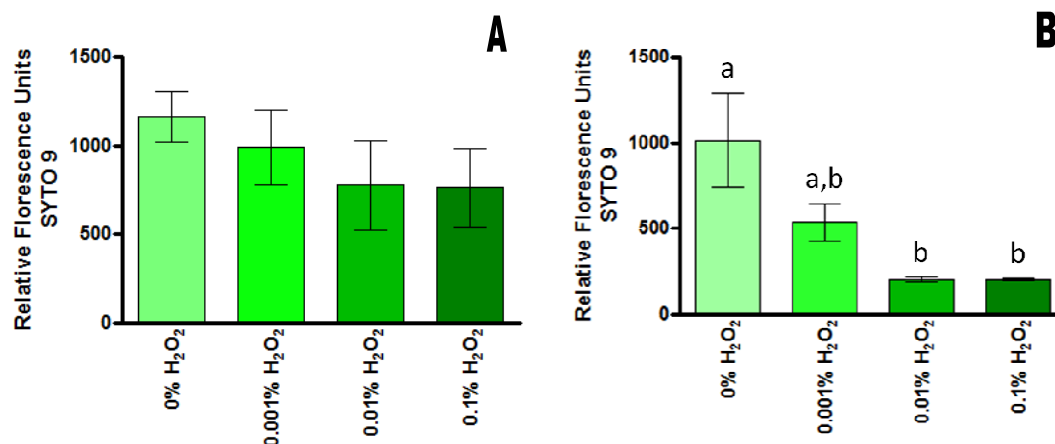


Figure 4-17: Hydrogen peroxide decreased the amount of viable cells in Mpt biofilms.

After twenty or thirty days of incubation in the presence of hydrogen peroxide, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). SYTO 9 staining represents the amount of viable cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.01$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

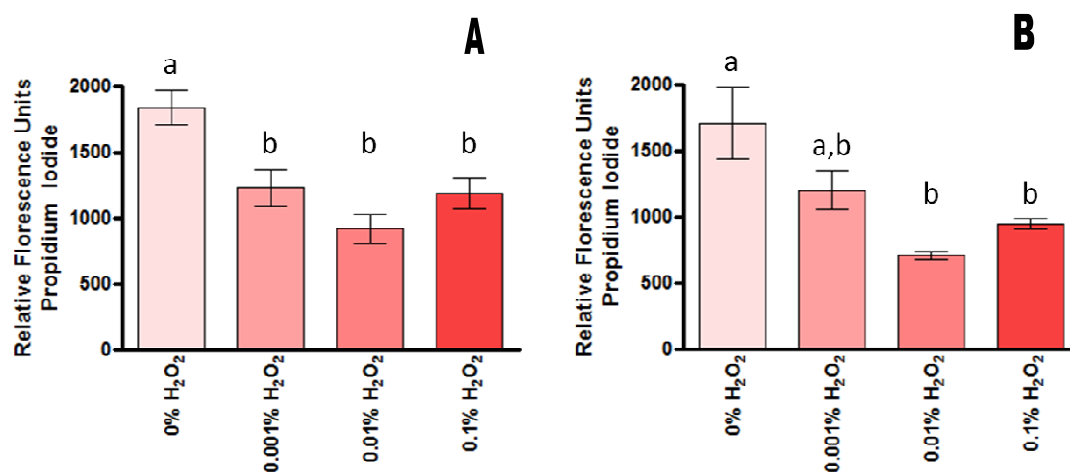


Figure 4-18: Hydrogen peroxide decreased the amount of dead cells in Mpt biofilms.

After twenty or thirty days of incubation in the presence of hydrogen peroxide, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). Propidium iodide staining represents the amount of nonviable cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.01$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

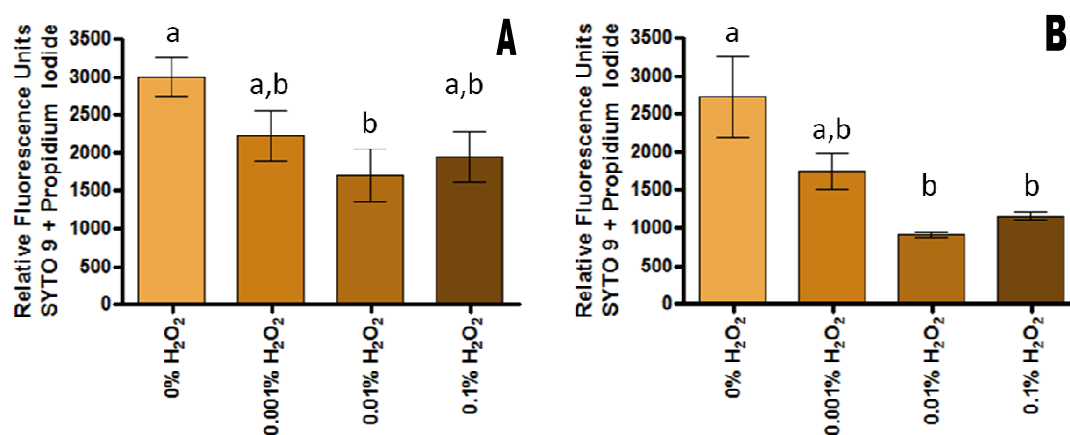


Figure 4-19: Hydrogen peroxide limited cellularity in biofilms. After twenty or thirty days of incubation in the presence of hydrogen peroxide, biofilms were stained with SYTO 9 and propidium iodide then read in a Fluoroskan FL fluorometer with excitation wavelength of 485 nm, emission wave lengths of 538 nm (green) and 635 nm (red). SYTO 9 and Propidium iodide staining represents the amount of viable and nonviable cells in a biofilm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

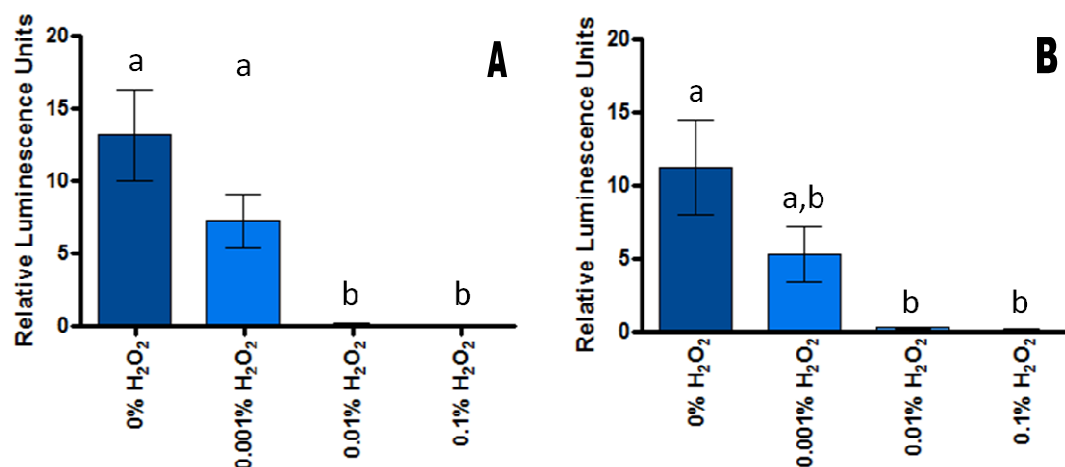


Figure 4-20: Hydrogen peroxide inhibited the metabolism of Mpt in biofilms. After incubation in media with or without hydrogen peroxide for twenty or thirty days, Mpt cells in biofilms were lysed and the amount of ATP was measured using a bioluminescence reaction and were read in a Fluoroskan FL luminometer. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

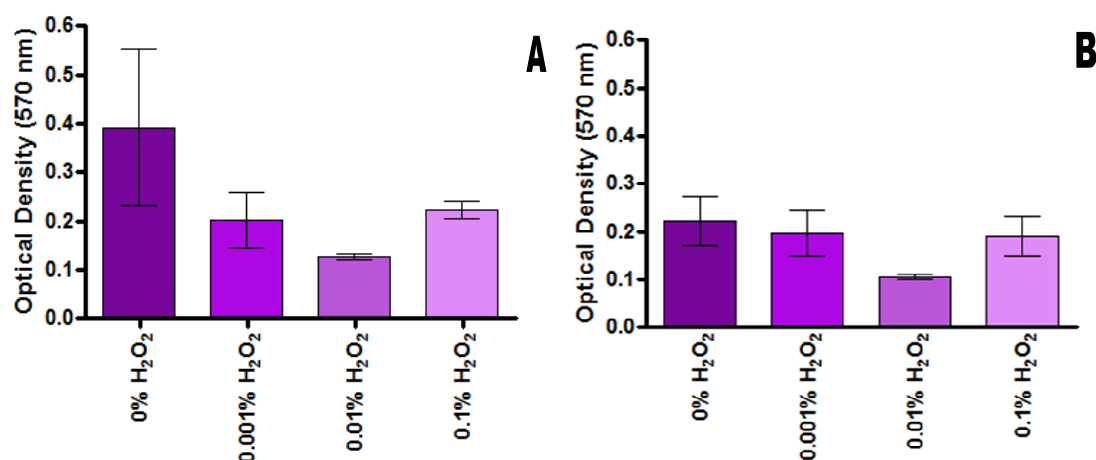


Figure 4-21: Hydrogen peroxide limited biomass development in Mpt biofilms. After incubation in media with or without hydrogen peroxide for twenty or thirty days, Mpt biofilms were stained with crystal violet then washed with deionized water. Crystal violet was solubilized with 95% ethanol, and then the crystal violet-ethanol solution was measured for optical density at 570 nm. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days. Error bars are standard error of the mean. Bars labeled with “a” or “b” are significantly different, $p < 0.05$. Bars labeled “a, b” are not significantly different than bar labeled “a” or “b”.

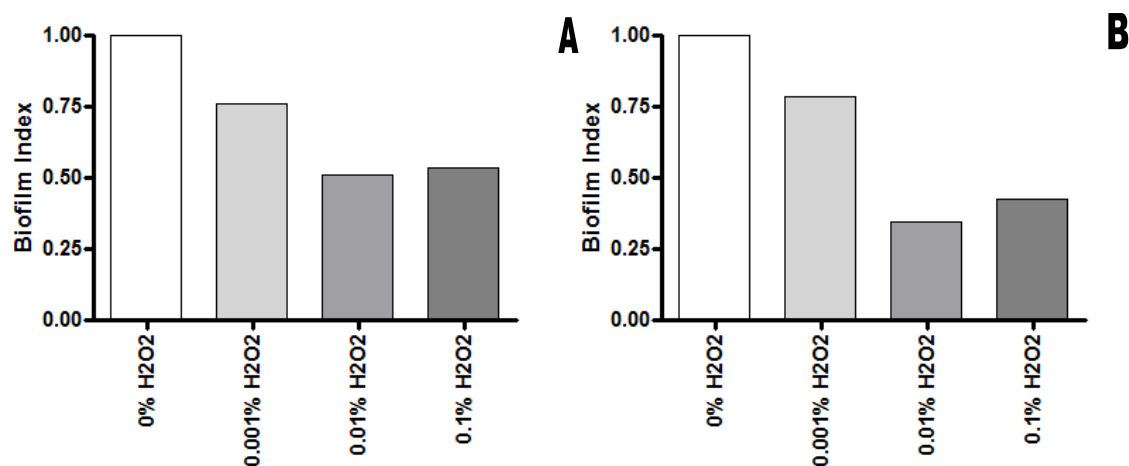


Figure 4-22: Hydrogen peroxide adversely affected biofilm development by Mpt. The biofilm index combines normalized data from viability ratio, cellularity, ATP and biomass assays. Data were normalized so that the mean for pH 6.8 equals 1. Panel A represents biofilms cultured for twenty days. Panel B represents biofilms cultured for thirty days.

Chapter 5: Discussion

Despite being dependent on exogenous mycobactin and being considered an obligate intracellular pathogen, Mpt can survive in the environment for weeks or months (54). The environment outside of a host is more hostile for Mpt than the inside of a host. In vivo, oxygen, carbon and iron are not plentiful but available. In the environment, essential nutrients may be limited and cells could be damaged by physical forces or caustic chemicals. An extracellular matrix could limit the impact of desiccation, physical forces or chemical damage, and thus provide a microenvironment for Mpt that limits the effects of stressful pH levels, changing osmotic pressure and antimicrobial chemicals. In short, biofilms may be a survival tactic that Mpt uses to endure in the environment until a new host is encountered (39).

This work used three different assays to describe the effects of pH stress, hypoxia and oxidative stress and on the formation of biofilms by Mpt. Each assay described slightly different aspects of Mpt biofilms. One assay alone would not have been sufficient to characterize the the biofilms. The viability assay used differential nucleic acid stains to describe the relative viability of cells in biofilms (17). The ATP assay used a bioluminescence reaction to measures ATP as an indicator of metabolic activity of cells in a biofilm (56). The biomass assay is a standard biofilm assay using crystal violet staining to describe biofilms by measuring the relative biomass (32). The Biofilm Index combined data from the viability, ATP and biomass assays to qualitatively describe which conditions produced the best biofilms.

The best biofilms produced by Mpt were in conditions that promoted the best growth of the organism. Biofilm formation does not appear to be a response by Mpt to a particular stress. Development of biofilms by Mpt was not enhanced and may have been inhibited by exogenous stress. Mpt formed the best biofilms in slightly acidic media and was inhibited in alkaline media. Oxidative stress from hydrogen peroxide did not promote cellular growth but may have promoted extracellular matrix formation. Oxygen deprivation inhibited biofilm formation. Since the growth requirements for Mpt are in a narrow range of pH and oxygen levels, Mpt is unlikely to form robust biofilms as a survival mechanism. However, Mpt may form small clusters of cells

surrounded by an extracellular matrix to extend their stay in the environment under these conditions.

Good agricultural hygiene practices may limit the spread of Mpt and other pathogens on the farm. Assuming that animals carrying Mpt can be identified, infected animals can be separated from healthy animals, either by physically segregating them or by culling infected animals. Currently the best practices entail keeping infected animals away from healthy animals; however, many apparently healthy animals may be Mpt carriers and may be shedding Mpt. If these apparently healthy animals did not spread Mpt in the environment, it is possible that Johne's disease would have been defeated long ago.

A diagnostic test with near perfect sensitivity for Johne's disease is unlikely to be developed in the very near future. Eradicating infected herds is extreme and not economically practical. Enhancements to agricultural practices are one strategy to defeat Mpt. The use of chemicals to kill Mpt in the buildings and in the fields may be a technique to limit the spread of Mpt. However, if Mpt is in a biofilm, the cells may be resistant to commonly used disinfectants.

The extracellular matrix and dead cells of a biofilm may limit penetration of disinfectants into a biofilm. Understanding the conditions that allow Mpt to form biofilms could result in more effective disinfection practices. New chemicals or new techniques to use current chemicals may enhance the effectiveness of destroying Mpt in place.

The Effects of pH on Biofilm Development

Robust Mpt biofilms formed in a narrow range of pH levels. Suboptimal pH conditions limited growth resulting in less developed biofilms. The extra stresses of nutrient acquisition, maintaining cell wall integrity and maintaining a proton gradient in suboptimal pH conditions may have inhibited growth. The pH of an environment can alter the availability of some essential ions such as iron. Suboptimal pH levels may require greater effort for maintenance of the cell wall as a result of the degradation of the cell wall. Under these conditions, Mpt may spend more energy in maintaining an electrochemical gradient than at optimal pH conditions, limiting the amount of energy that could be used for growth and maintenance.

Had only the biomass assay been used, we would have assumed that alkaline conditions promoted Mpt biofilm formation. The viability and ATP assays however refuted that claim. With the viability assay, alkaline conditions resulted in a low viability ratio and low cellularity. The ATP assay demonstrated that there was very little metabolic activity in alkaline conditions. Slightly acidic environments produced the best biofilms in terms of cellularity, viability and metabolic activity. Mpt grew well in M7H9C at pH 6.8, but did not grow well or form robust biofilms in alkaline conditions.

The pH level of the medium in which Mpt biofilm was cultured affected the biomass assay in an unexpected way. Apparent biofilms that formed in alkaline conditions had higher optical densities than those formed in neutral and acidic conditions. Macroscopically, however, biofilms cultured in alkaline conditions were obviously less robust than biofilms cultured in neutral or acidic media. Furthermore, microscopically, there were few cells present in the biofilms cultured in alkaline media. Since Mpt had low viability and low cellularity compared to more acidic conditions, it is unlikely that Mpt formed more biomass under alkaline conditions than under acidic conditions.

There may have been some interactions among Mpt, crystal violet, Thermanox™ discs and/or M7H9C in alkaline conditions that increased the amount of crystal violet bound to the samples. Perhaps under alkaline conditions, more crystal violet may have been able to penetrate Mpt cells. The proprietary plastic properties of Thermanox™ may have been altered in alkaline conditions enabling more crystal violet to bind to the disc than in neutral conditions. Finally, the bovine serum albumin in M7H9C may have served to bind crystal violet to the Thermanox™ plastic.

The color and intensity of crystal violet is also affected by pH level. In very acidic conditions crystal violet turns yellow. In very alkaline conditions, crystal violet turns colorless. These properties are due to the protonation level of crystal violet and may have affected the absorption of 570 nm light.

Iron and pH Level

One of the reasons Mpt may not have thrived in alkaline conditions relates to iron availability. Iron is an essential element for Mpt metabolism. Mpt lacks the ability to form mycobactin, which is involved in iron acquisition. Studies have shown that in acidic environments, Mpt is capable of growing without mycobactin, indicating there may be a secondary iron acquisition mechanism (31). Since mycobactin binds ferric (Fe^{3+}) not ferrous (Fe^{2+}) iron, Mpt may use ferrous iron in acidic environments (42).

The pH level of an environment may influence the species of iron in the environment. In neutral solutions, ferrous iron is soluble but ferric iron is nearly insoluble. In neutral pH conditions and in the presence of oxygen, iron is predominantly in the ferric state because ferrous iron is quickly converted to ferric iron. In acidic conditions, ferrous iron has a higher availability than at a neutral pH level; however ferric iron still dominates. In alkaline conditions there is very little ferrous iron available. Ferric iron may not exist as a dissociated ion, and therefore may not be available to bind to mycobactin (48). In alkaline conditions, ferric iron ions are limited which may limit Mpt growth. In acidic conditions, ferric ions are not as limited and more ferrous iron ions are available.

Environmental pH levels

Soils can have a wide range of pH levels. There is some evidence that Mpt is less capable of surviving in alkaline soils than in acidic soils. W. D. Richards cited several observations that supported this claim. In New York, several counties with alkaline soils had been free of Johne's disease for years, while nearby counties with acidic soils had chronic difficulty with Johne's disease. In England, farms situated on alkaline soils had no cases of Johne's disease, but nearby farms on acidic soils had some cases of Johne's disease (38).

Richards went on to describe the efforts of a farmer in Missouri, who had previously had a herd free of Johne's disease, but acquired a calf that was infected with Mpt. Johne's disease soon spread to several other animals in the herd. Through judicious application of hydrated lime, which alkalized the soil and proper farm management, Johne's disease was eliminated from the farm (38).

If Mpt uses biofilms as a survival tactic, the optimal pH conditions must fall in a narrow range of acidity between pH 7.0 and pH 6.5. Consistent with Richards, alkaline conditions inhibited Mpt cellularity, viability and metabolic activity, which limited biofilm development. Further investigation into why the crystal violet assay did not agree with the viability and ATP assays will need to be conducted. In view of this, Mpt is unlikely to produce biofilms in environments with alkaline or very acidic conditions.

The Effects of Oxygen Deprivation on Biofilm Development

Subjecting Mpt to hypoxic conditions inhibited the growth of the organism independent of the concentration of Oxyrase™ used to produce hypoxic conditions. Even though cells cultured in media with 1/32 Oxyrase™ had one-eighth of the enzymes and NADH of 1/4 Oxyrase™, both concentrations of Oxyrase™ resulted in similar cellularity, viability, biomass and ATP concentrations. Lower concentrations of Oxyrase™ resulted in less hypoxic conditions and for a shorter period of time. Oxyrase™ did not maintain hypoxic conditions for the duration of incubation, and the duration of hypoxic conditions was proportional to the Oxyrase™ concentration. Mpt incubated with 1/32 Oxyrase™ should have grown better than with 1/4 Oxyrase™. However, Oxyrase™ may have influenced Mpt to adopt a stationary phase or dormant phenotype early and the concentrations of Oxyrase™ were immaterial.

A pilot study was conducted to describe how the concentration of Oxyrase™ affected oxygen concentration of a liquid over time. The oxygen content diminished more rapidly with a higher concentration of Oxyrase™ than a lower concentration of Oxyrase™. A lower concentration of Oxyrase™ reduced the oxygen content less than a higher concentration of Oxyrase™. The liquid remained hypoxic for a shorter period of time with a lower dose of Oxyrase™ than with a higher dose of Oxyrase™. Extrapolating from the pilot study, hypoxic conditions likely existed for less than fourteen days during the twenty or thirty days of incubation. Since Mpt is an aerobic organism, a sudden reduction in oxygen concentration likely killed some of the cells and limited the metabolic activity of the surviving cells. However, when oxygen levels returned to a level that allowed respiration, Mpt should have begun to grow.

Cells incubated in a medium with 1/32 Oxyrase™ should have resumed growing before cells growing in medium with 1/4 Oxyrase™. If cells incubating in a medium with 1/32 Oxyrase™ began growing before cells growing in a medium with 1/4 Oxyrase™, then we should have observed a difference in the cellularity, viability, biomass and ATP levels. However, after twenty or thirty days of incubation, Mpt biofilms had similar cellularity, biomass, viability, and ATP levels regardless of the concentration of Oxyrase™ used.

Cells cultured without Oxyrase™ had much higher cellularity, viability, biomass and ATP levels than cells cultured in the presence of Oxyrase™. Hypoxic conditions may have killed off a portion of the cells, and delayed exponential growth for the remaining viable cells. Very few cells were viable after exposure to hypoxic conditions for 20 or 30 days. Even if oxygen deprivation killed some of them the growth should have been rebounding after twenty days, however, after 30 days, viability continued to be low. It is possible that hypoxic conditions caused early onset of a stationary phase phenotype or perhaps a dormant phenotype.

The decline in oxygen content is different between the natural processes in vivo and with Oxyrase™. In the intestinal tract, oxygen levels decline slowly as cells transition from the mouth to the colon. Oxyrase™ reduces the oxygen content quickly. Conversely, the oxygen levels increase rapidly as cells are egested and increase slowly in the presence of Oxyrase™.

As Mpt descends into the colon, oxygen levels lower, becoming more hypoxic. When egested into the environment, the interior of animal scat is generally anoxic or hypoxic. Over time, conditions become less hypoxic. If Mpt were to employ biofilms as a survival tactic, the conditions near the exterior of fecal matter may allow Mpt to form a biofilm, i.e. where conditions are not hypoxic.

The Effects of Oxidative Stress on Biofilm Development

During an investigation of oxidative stress response in *M. avium* by Geier et al, biofilms cultured in the presence of hydrogen peroxide developed more biomass than biofilms cultured without hydrogen peroxide (19). Much of the intent for our experiment, exposing Mpt to hydrogen peroxide, was to apply their work with *M. avium* to Mpt. Several aspects of the protocols they

used were altered, which limited direct comparison of the data. While the strain of *M. avium* used by Geier et al may have produced more biomass when exposed to hydrogen peroxide, *M. paratuberculosis* PAMSUM 8 did not respond in a similar fashion.

Oxidative stress reduced cellularity, viability and metabolic activity in Mpt biofilms. However, biomass was not affected. Similar optical densities from the biomass assay when Mpt was cultured with or without hydrogen peroxide indicate that oxidative stress may promote extracellular matrix production in Mpt. Although at twenty days of incubation, Mpt exposed to hydrogen peroxide had slightly less biomass than Mpt exposed to hydrogen peroxide the biomass was statistically similar ($p > 0.05$). After thirty days of incubation Mpt not exposed to hydrogen peroxide had similar levels of biomass as Mpt exposed to hydrogen peroxide ($p > 0.05$).

Oxidative stress may have induced phenotypic changes in exponentially growing Mpt that improved viability over exponentially growing Mpt in the absence of oxidative stress. After twenty days, Mpt cultures initiated with 0.001% or 0.01% hydrogen peroxide had higher viability ratios than Mpt cultured without hydrogen peroxide. However, after thirty days Mpt biofilms cultured with hydrogen peroxide had lower viability ratios than Mpt biofilms cultured without hydrogen peroxide. The phase of growth may help explain the change in viability from twenty to thirty days. Exposure to hydrogen peroxide may have induced early onset of stationary phase in Mpt. Phenotypically, Mpt changes as it transitions from exponential growth to stationary phase including cessation of cellular division and production of osmoprotectants. While Mpt biofilms had a higher proportion of viable cells after twenty days of exposure to 0.01% or 0.001% hydrogen peroxide than when not exposed to hydrogen peroxide, SYTO 9 staining indicated that biofilms exposed to hydrogen peroxide had fewer viable cells than biofilms not exposed to hydrogen peroxide. Without hydrogen peroxide Mpt grew better, but with hydrogen peroxide Mpt had a higher proportion of viable cells.

Growing in the absence of hydrogen peroxide may have allowed Mpt to allocate more energy to cell division than Mpt exposed to hydrogen peroxide. Mpt may have been spending more energy resisting oxidative stress when in the presence of hydrogen peroxide. Possibly under the stress of hydrogen peroxide, Mpt shut down and went into a dormant state some time

before day twenty. The viability assay indicated that there were some viable cells and macroscopically biofilms formed. Cells must have survived at least for a while in 0.01% H₂O₂ or 0.1% H₂O₂.

While the growth of Mpt was inhibited by hydrogen peroxide, our results suggest that oxidative stress may have promoted extracellular matrix formation. If Mpt forms an extracellular matrix in response to oxidative stress, such as transitioning from an anaerobic to aerobic environment, the extracellular matrix may help Mpt survive and adjust to aerobic conditions.

Biomass Assay

A typical biofilm assay for many researchers uses crystal violet to measure biomass. Fletcher, O'Toole et al, Carter et al and Geier et al used the biomass assay to great effect in measuring biofilms (5, 18, 19, 33). We found biofilm or biofilm-like formations that were not firmly attached to the substrate. With our system, after biofilms were stained with crystal violet, some of what appeared to be biofilm sloughed off during the washing process. If biofilms are defined as communities of cells bound together in an extracellular matrix, and not necessarily to a surface, some of the community was lost during the washing process. We confined our observations to cells bound to the surface. Biofilms were defined as being bound to each other and to a surface. Cells that sloughed off during the washing process were disregarded.

Biofilms produced by Mpt may be only surface-associated and only weakly attached to the surface. Firmer attachment of cells to the substrate may be provided by some biological or physical factor that was not included in our system. Initial attachment is likely through hydrophobic interaction with the surface, which also plays a role in cells-cell adhesion (57). Thermanox coverslips are coated on one side, likely through a molecular charge to promote adhesion of eukaryotic cells. After twenty days, many cells in a well were in apparent biofilm structures; however, as coverslips were removed from the wells, large portions of the apparent biofilm were left in the well. The Thermanox plastic may have limited Mpt cell adhesion; however our observation indicated that at least a portion of the cells did adhere to the cells. Possibly another substrate would have given different results.

The amount of cells remaining behind after washing was highly variable using our system. Optical density readings were similar within a treatment; however there were a few outlying data points that increased the variance in the optical densities of treatments. Certainly part of the variance was a result of washing technique; however every effort was made to wash replicates identically. It is also possible that part of the variance may have been due to the Thermanox™ discs occasionally being up-side-down, which because they are charged on one side affected biofilm formation.

Viability Assay

The viability assay used propidium iodide and SYTO 9 to differentially stain cells according to their viability. Data from SYTO 9 and propidium iodide staining provided a profile of the relative amounts of live cells and dead cells in a biofilm. SYTO 9 data described the relative amount of viable cells in the sample. Propidium iodide data described the relative amount of non-viable cells, i.e. dead, damaged and dying cells in the sample. Data from viability staining were combined in several ways. A viability ratio, calculated by dividing the SYTO 9 signal by the propidium iodide signal for each replicate, indicated the relative ratio of live cells to dead cells. Summing data from SYTO 9 and propidium iodide for each replicate described the relative amount of cells, both living and dead in a sample, or cellularity.

There was an apparent effect of time on viability for all experiments indicating that there were fewer viable cells present at thirty days than at twenty days. Further, SYTO 9 intensity and cellularity was significantly lower at thirty days for the hydrogen peroxide experiment and propidium iodide staining was significantly lower at thirty days for the hypoxia experiments. However there was no effect of time on the ATP levels for any experiment. If it is assumed that the fluorochromes used stained only nucleic acids, then the amount of ATP per cell must be greater at thirty days than at twenty days. Given that Mpt is in stationary phase at thirty days, this is unlikely. Both propidium iodide and SYTO 9 are essentially hydrophobic molecules and may associate with structures other than nucleic acids in mycobacteria, such as long chain mycolic

acids in the cell wall or the short chain mycolates believed to comprise the extracellular matrix in mycobacterial biofilms (57)

The viability ratio described the relative ratio of live to dead cells, but did not describe how many live or dead cells were present in the sample. A high viability ratio by itself did not constitute a robust biofilm but merely described what proportion of cells in the sample was viable. A biofilm with few cells present could have a higher viability ratio than a biofilms that was visually robust.

Combined, the data from these two stains described cellularity. In some respects cellularity was a better measure of the robustness of a biofilms than was the viability ratio. Under some conditions cells may not have grown well, but were still viable and had a high viability ratio.

If biofilms were simply a monolayer of cells, the fluorochromes used in the viability assay would all be excited equally and emit at the same intensity. Since biofilms are three-dimensional, the excitation light must pass through exterior cells to reach interior cells and emitted fluorescence from interior cells must pass through the exterior cells to be detected. The fluorescence in more mature biofilms may be underestimated with the techniques used.

ATP Assay

The ATP assay described metabolic activity through a bioluminescence reaction that can be used to measure ATP concentration. Some researchers have used BacTiter Glo™ to count the number of cells in a sample, for instance in minimum bactericidal concentration experiments (56). The ATP assay measures the amount of ATP for the whole sample. The ATP in a sample is governed by several variables, including the number of cells, the viability of cells and the metabolic state of the cells. Some cells may be dead and have no ATP. Some cells may be in a dormant or stationary state and have minimal ATP. Some cells may be actively metabolizing and have large amounts of ATP per cell.

The metabolism of cells will change based on growth conditions and the phase of growth. During lag phase the ATP concentration present may be very high in viable cells even though there are few cells; however, during stationary phase there may be plenty of cells but very little

ATP in each cell. If all cells in different treatments had the same amount of ATP, then the number of cells could be indirectly measured by measuring ATP. In contrast, if each sample had the same amount of cells, the amount of ATP would be indicative of the metabolic state of the population.

Three problems arose when the ATP assay was applied to Mpt biofilms. First, few cells were present in some samples. The second problem involved the extracellular matrix encasing the biofilm cells. The third obstacle was the mycobacterial cell wall. Together these obstacles limited the resolution achieved with the ATP assay

In some conditions there were few cells adhering to the substrate discs. Since there were few cells, the amount of ATP may have been below the lower threshold to measure. Generally, the cellularity of a treatment corresponded with the amount of ATP present. Conditions resulting in well formed biofilms typically had high amounts of ATP present.

The extracellular matrix also may have limited the access of the lytic agents to the cells. Determining just how much the extracellular matrix affected cell lysis was difficult to measure. Lysozyme in conjunction with BacTiter Glo™ was no better than BacTiter Glo™ alone at room temperature. However, lysozyme in conjunction with rapid freeze-thaw cycles and BacTiter Glo™ resulted in the best luminescence.

BacTiter Glo™ was designed to lyse Gram-positive and Gram-negative cells typically used in research. *Mycobacterium* species have a sturdier cell wall and do not lyse as easily. The mycolic acid in the cell wall hindered the lysis of cells and limited the liberation of ATP from cells. A rapid freeze-thaw cycle between -80° C and 37° C was moderately successful in lysing cells and increasing the bioluminescence generated with BacTiter Glo™.

Nevertheless, cell lysis may have been incomplete even with lysozyme and freezing, which may have limited the amount of ATP available to luciferase. Since all of the biofilms were treated the same during the ATP assay, the relative amounts of ATP would have been proportional to what may have been optimum. Had there been more cells or the cells lysed more efficiently the luminescence from the luciferin reaction may have been brighter and given the assay better contrast.

Biofilm Index

Each of the assays used to describe Mpt biofilms described different aspects of biofilms. In order to bring these different aspects into one picture, a qualitative biofilm index was developed. The data from each assay were in different scales. The optical density from biomass ranged from zero to four, where zero was perfectly clear and four was perfectly opaque. The ATP assay gave results from zero to hundreds of relative luminescence units. Since the scales of data did not match, data was normalized so that each assay had equal weight. The biofilm index qualitatively described which conditions had the best biofilms.

Closing Comments

This study looked at three physiologically relevant variables that may influence biofilm formation by Mpt. Biofilms may allow Mpt to resist changes in pH and oxidative stress that could allow Mpt to be virulent. Three assays allowed to better define and characterize biofilms than could a single assay could alone.

The formation of a biofilm may be sufficient to allow Mpt to pass from one host to another. Mpt may use biofilm in conjunction with other survival techniques such as an intermediate host and dormancy to endure until a new host is encountered.

In some respects the design of these experiments does not mimic actual conditions. In vivo Mpt has no access to mycobactin and uses an unknown iron acquisition pathway. In the environment, Mpt has no endogenous mycobactin. Outside of the host Mpt is likely to be incapable of growth. One of the assumptions of these experiments was that growth was necessary for biofilm development. Without mycobactin, Mpt is incapable of growing in vitro. Perhaps one day we will be able to mimic the in vivo conditions and culture Mpt without mycobactin.

Hypoxic conditions may have induced a phenotypic change in Mpt to allow Mpt to survive. Mpt may switch to a dormant phenotype to survive as oxygen levels become more hypoxic. Lack of oxygen is known to induce dormancy in *Mycobacterium smegmatis* (13)

A likely follow up to these experiments would be to remove the assumption that Mpt must grow to form a biofilm. By starting cultures with a heavy inoculum, on the order of 10^8 to 10^{10} CFU/ml, biofilms may develop more quickly. One hazard with this technique and why we used 10^5 CFU/ml is that contamination is not apparent with such heavy inocula.

Mpt may not adhere to surfaces when it forms biofilms. Mpt may be associated with a surface but not adhere to it as strongly as other bacteria species. In cell culture flasks and in the 24 well plates much of the biomass of the biofilms was not firmly attached to the surface. Mpt forms ribbons of biofilm. The next step in removing that assumption is to use large pore filters to capture cells that adhere to each other but not to surfaces.

List of Figures

Figure 3-1: Cut-away view of the culture chamber.	25
Figure 3-2: Dilution Diagram for Mpt biofilms cultured in M7H9C Δ pH	26
Figure 3-3: Dilution Diagram for Mpt biofilms cultured in M7H9C with Oxyrase.....	27
Figure 3-4: Dilution diagram for Mpt biofilms cultured in MSUM1 with hydrogen peroxide	28
Figure 3-5: Change in luminescence over time.	29
Figure 4-1: Alkaline conditions inhibited the viability of Mpt in biofilms.	43
Figure 4-2: Alkaline conditions limited the amount of viable Mpt cells in biofilms.	44
Figure 4-3: Alkaline conditions limited the amount of nonviable Mpt cells in biofilms.	45
Figure 4-4: Alkaline conditions limited the cellularity of Mpt biofilms.....	46
Figure 4-5: Alkaline conditions limited the amount of ATP present in Mpt biofilms	47
Figure 4-6 Alkalinity caused an apparent increase in biomass.....	48
Figure 4-7: Alkaline conditions inhibit biofilm development	49
Figure 4-8: Oxyrase™ reduces the oxygen concentration but cannot maintain hypoxic conditions indefinitely.	50
Figure 4-9: Hypoxic conditions limited viability of Mpt when cultured with Oxyrase™.	51
Figure 4-10: Hypoxic conditions limited the amount of viable Mpt when cultured with Oxyrase™.	52
Figure 4-11: Hypoxic conditions limited the amount of nonviable Mpt when cultured with Oxyrase™.	53
Figure 4-12: Hypoxic conditions limited cellularity of Mpt when cultured with Oxyrase™.	54
Figure 4-13: Hypoxic conditions inhibited metabolic activity of Mpt when cultured with Oxyrase™.	55
Figure 4-14: Hypoxic conditions resulted in an apparent increase in biomass of Mpt biofilms when cultured with Oxyrase™.	56
Figure 4-15: Hypoxic conditions inhibited growth of Mpt when cultured with Oxyrase™ for 20 days.....	57
Figure 4-16: Hydrogen peroxide diminished the viability of Mpt.....	58
Figure 4-17: Hydrogen peroxide decreased the amount of viable cells in Mpt biofilms.	59
Figure 4-18: Hydrogen peroxide decreased the amount of dead cells in Mpt biofilms.....	60
Figure 4-19: Hydrogen peroxide limited cellularity in biofilms.....	61
Figure 4-20: Hydrogen peroxide inhibited the metabolism of Mpt in biofilms.	62
Figure 4-21: Hydrogen peroxide limited biomass development in Mpt biofilms.	63
Figure 4-22: Hydrogen peroxide adversely affected biofilm development by Mpt.....	64

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